THE ROLE OF GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 1 IN MITOCHONDRIAL PHOSPHOLIPID BIOSYNTHESIS OF COLD-BODIED FISHES

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THE ROLE OF GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 1 IN MITOCHONDRIAL PHOSPHOLIPID BIOSYNTHESIS OF COLD-BODIED FISHES

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ABSTRACT

Mitochondrial biogenesis is induced by low temperature in many fish species. For example, cold acclimation of *Gasterosteus aculeatus* (threespine stickleback) increases mitochondrial densities in oxidative skeletal muscle. Oxidative muscles of Antarctic icefishes (suborder Notothenioidei) also have high mitochondrial densities characterized by higher densities of phospholipids compared to red-blooded notothenioids. Mitochondrial biogenesis has been well studied in mammals yet it is unknown how mitochondrial phospholipid synthesis is regulated. I hypothesized that both activity and mRNA levels of glycerol-3-phosphate acyltransferase (GPAT), the rate-limiting enzyme in glycerolipid biosynthesis, would increase in oxidative muscle of stickleback, where mitochondrial biogenesis occurs, but not in liver, in response to cold acclimation, and that GPAT1 and /or GPAT2 mRNA levels would be higher in hearts of icefishes compared to red-blooded species. To test these hypotheses, maximal activity of GPAT and mRNA levels of GPAT1 and GPAT2 were measured in liver and oxidative muscle of cold- and warm- acclimated stickleback. GPAT1 and GPAT2 mRNA levels were also quantified in hearts and livers of red- and white-blooded Antarctic notothenioids. Additionally, cDNA of GPAT1 was sequenced in Antarctic and sub-Antarctic notothenioids to gain insight to the evolution of a mitochondrial membrane protein and identify candidate amino acid residues responsible for maintaining function at cold temperature. GPAT activity increased in oxidative muscle but not in liver, and transcript levels of GPAT1 increased in liver but not in oxidative muscle, in response to cold acclimation in stickleback. GPAT2 transcripts were undetectable in both tissues. GPAT1 mRNA levels were highest in liver of red-blooded Antarctic notothenioids and did not differ in hearts between red- and white-blooded fishes, and GPAT2 transcripts were undetectable. GPAT protein levels may not change concurrently with GPAT1 and GPAT 2 mRNA levels because GPAT3 or 1-acylglycerol-3-phosphate acyltransferase (AGPAT), the enzyme subsequent to GPAT, may be involved in regulating phospholipid synthesis during mitochondrial biogenesis. The amino acid sequence of GPAT1 is highly conserved (97.94-98.06%) among Antarctic and sub-Antarctic notothenioids, with three potential sites in the cytosolic region that may be important for maintaining function at cold temperature: Ser415Ala, Asp603Glu and Thr648Ala.

TABLE OF CONTENTS

Page

Signature Page ..... **i**

Title Page ..... **iii**

Abstract ..... **v**

Table of Contents ..... **vii**

List of Figures ..... **xi**

List of Tables ..... **xiii**

List of Appendices ..... **xv**

Acknowledgements ..... **xvii**

Dedication ..... **xviii**

General Introduction **1**

General Introduction References **5**

Chapter 1. Characterization of GPAT1 in Antarctic notothenioid fishes **9**

1.1 Abstract **9**

1.2 Introduction **10**

1.3 Materials and Methods **13**

1.3.1 Tissue collection 13

1.3.2 RNA isolation and cDNA synthesis 13

1.3.3 Housekeeping gene analysis 14

1.3.4 Measuring GPAT1 and GPAT2 transcript levels 14

1.3.5 Sequencing GPAT1 and GPAT2 cDNA 15

1.3.6 Statistical analysis 16

1.4 Results **17**

Page

1.4.1 Housekeeping gene analysis 17

1.4.2 GPAT1 and GPAT2 transcript levels 17

1.4.3 GPAT1 cDNA sequence 17

1.5 Discussion 19

1.6 Acknowledgements 23

1.7 Figures 24

1.8 Tables 59

1.9 References 66

Chapter 2. Molecular Drivers of Mitochondrial Membrane Proliferation in response to cold acclimation in threespine stickleback 71

2.1 Abstract 71

2.2 Introduction 72

2.3 Materials and Methods 73

2.3.1 Animal care 73

2.3.2 Maximal GPAT activity of crude liver homogenate 74

2.3.3 Gene expression 75

2.3.4 Statistical analyses 76

2.4 Results 77

2.4.1 Physical characteristics 77

2.4.2 Apparent Michaelis Menten constant 77

2.4.3 Maximal GPAT activity in fresh and frozen tissue 77

2.4.4 Substrate preference 78

2.4.5 GPAT activity in warm- and cold- acclimated stickleback 78

2.4.6 Expression of genes involved in lipid biosynthesis 78

Page

2.5 Discussion **79**

2.5.1 Characterization of GPAT activity 79

2.5.2 Molecular drivers of membrane biosynthesis 80

2.6 Acknowledgements **82**

2.7 Figures **83**

2.8 Tables **89**

2.9 References **92**

Appendices **94**

General Conclusion **97**

General Conclusion References **100**

LIST OF FIGURES

Page

Chapter 1 24

Fig. 1.1: GPAT1 mRNA levels in heart and liver of notothenioids 24

Fig. 1.2: Predicted cDNA sequence of GPAT1 in three notothenioid species 25-36

Fig. 1.3: GPAT2 partial cDNA sequence obtained in Antarctic notothenioids 37-41

Fig. 1.4: Amino acid sequence alignment of fish, bird, and mammalian GPAT1 42-57

Fig. 1.5: dN/dS ratios in GPAT1 of selected fish species 58

Chapter 2 83

Fig. 2.1: Lineweaver-Burk plot of GPAT activity in fresh liver tissue 83

Fig. 2.2: Maximal GPAT activity in *G. aculeatus* fresh and frozen liver 84

Fig. 2.3: Fatty acyl substrate preference of GPAT in *G. aculeatus* 85

Fig. 2.4: GPAT activity in pectoral adductor and liver tissue of cold-acclimated *G. aculeatus* . 86

Fig. 2.5: Transcript levels of genes involved in membrane biosynthesis in liver 87

Fig. 2.6: Transcript levels of genes involved in membrane biosynthesis in oxidative muscle 88

Fig. 2.A-1: GPAT1 activity is linear over time 94

Fig. 2.A-2: GPAT1 activity is proportional to the amount of protein 95

LIST OF TABLES

Page

Chapter 1 59

Table 1.1: Gene-specific primers used to quantify relative transcript levels 59

Table 1.2: Degenerate and gene-specific primers used to obtain GPAT1 and GPAT2 cDNA ... 60

Table 1.3: Housekeeping gene analysis 61

Table 1.4: Active site motifs of GPAT1 62

Table 1.5A: Transmembrane domain I of GPAT1 63

Table 1.5B: Transmembrane domain II of GPAT1 64

Table 1.6: Nonsynonymous amino acid substitutions in GPAT1 orthologs 65

Chapter 2 89

Table 2.1: Sequences of primers used for qRTPCR 89

Table 2.2: Effects of cold temperature on physical characteristics (GPAT assays) 90

Table 2.3: Effects of cold temperature on physical characteristics (mRNA) 91

LIST OF APPENDICES

Page

2.A Supplemental Figures.....**94**

2.B Permission of coauthors for publication.....**96**

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DEDICATION

I would like to dedicate my thesis to my sons, Isaac and Seth. Thank you.

GENERAL INTRODUCTION

Cold temperature decreases biochemical reaction rates, including those carried out by aerobic metabolic enzymes. Because fish are ectotherms, their body temperature is the same as their environment, making them especially susceptible to the negative effects of cold temperature. Maintaining ATP production can be particularly challenging for fish at cold temperature. Yet, some fishes endure seasonal declines in temperature as great as 20 °C, and thrive in cold waters (O'Brien, 2011).

Several temperate fish species maintain the production of ATP at low temperature by increasing the concentration of rate-limiting aerobic metabolic enzymes in oxidative tissues (Shaklee et al., 1977; Somero, 2004). Many of these enzymes are localized to the mitochondrion, therefore increases in their concentration are accompanied by an increase in mitochondrial density in oxidative muscle (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010; Tyler and Sidell, 1984). For example, the percent cell volume occupied by mitochondria increases 1.9-fold in oxidative skeletal muscle in response to cold acclimation in *Gasterosteus aculeatus*, the threespine stickleback (Orczewska et al., 2010).

Polar fishes also have high densities of mitochondria and Antarctic Channichthyidae, or icefishes, from the suborder Notothenioidei, have extraordinarily high mitochondrial densities in oxidative muscle (O'Brien and Mueller, 2010). Icefish are known for their lack of expression of the circulating oxygen-binding protein hemoglobin (Hb) as adults (Ruud, 1954). The percentage cell volume displaced by mitochondria is 51% in the oxidative swimming muscle, pectoral adductor, of the icefish *Champsocephalus esox,* compared to 24.9% in the red-blooded species *Gobionotothen gibberifrons*, and as high as 36.5% in cardiac myocytes of icefish *C. aceratus* compared to 15.9% in the red-blooded notothenioid *G. gibberifrons* (Johnston et al., 1998; O'Brien and Mueller, 2010; O'Brien and Sidell, 2000; O'Brien et al., 2003). Typically the percentage of the cell volume occupied by mitochondria is positively correlated with aerobic metabolic capacity but this is not the case for icefishes. Instead, high densities of mitochondria are thought to be crucial for enhancing oxygen diffusion (Runswick et al., 1989; Smotkin et al., 1991).

Mitochondrial biogenesis has been well studied in mammals, however little is known about how it is regulated in fish. Peroxisome proliferator-activated receptor gamma coactivator I alpha (PGC-1α) is considered the master regulator of mitochondrial biogenesis in mammals because it coordinates the expression of nuclear and mitochondrial gene products targeted to the mitochondrion, and controls replication of the mitochondrial genome (Puigserver et al., 1998). While much is known about how mitochondrial proteins are synthesized and how mitochondrial DNA is replicated, little is known about how phospholipid synthesis is regulated in mammals (or fishes) during mitochondrial biogenesis. However, the increase in mitochondrial phospholipids is likely attributable to an upregulation of one or more enzymes in the glycerolipid synthesis pathway (Bremer et al., 2012; O'Brien and Mueller, 2010; Scarpulla, 2008).

Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first and committed step in glycerolipid synthesis, making it a candidate gene for regulating phospholipid synthesis in mitochondrial biogenesis (Dircks and Sul, 1997). It catalyzes the synthesis of lysophosphatidic acid (LPA) from glycerol-3-phosphate (G3P) and fatty acyl-CoA. There are at least four GPAT isoforms (GPAT1-4) in mammals differing in their subcellular location, sensitivity to the sulfhydryl reagent N-ethylmaleimide (NEM), and fatty acyl-CoA preference (Gimeno and Cao, 2008; Takeuchi and Reue, 2009; Wendel et al., 2009). GPAT1 and GPAT2 are mitochondrial isoforms located in the outer membrane and GPAT3 and GPAT4 are localized to the endoplasmic reticulum (ER) membrane (Cao et al., 2006; Coleman and Lee, 2004). Genome sequences of the model fish species *Gasterosteus aculeatus*, *Takifugu rubripes* and *Danio rerio* predict at least three GPAT isoforms in fish, similar to mammalian GPAT1, GPAT2 and GPAT3.

Both mitochondrial and microsomal GPAT isoforms are transmembrane proteins and, in the case of cold-bodied fishes, likely interact with membranes rich in polyunsaturated fatty acids (PUFAs). Cold temperature decreases membrane fluidity, and to maintain membrane function, cold- adapted and cold-acclimated fishes increase the relative proportion of PUFAs in the process known as homeoviscous adaptation (Crockett and Hazel, 1995). Changes in membrane composition may be correlated with changes in amino acid sequence within transmembrane domains of membrane-bound proteins (Porta et al., 2013).

As an integral membrane protein, I hypothesized that as a result of mutation and natural selection, the GPAT1 amino acid sequence in Antarctic notothenioids may have evolved to maintain protein function at cold temperature. Synthesizing more enzymes can be energetically expensive, therefore species living in constantly cold environments have evolved enzymes that maintain catalytic efficiency at cold temperatures (Hochachka and Somero, 2002). These enzymes are characterized by decreased conformational stability, increased flexibility and highly conserved active sites, compared to orthologs from warm-bodied species (Crawford et al., 1989; Fields and Somero, 1998). Many studies have shown that proteins can adapt to function at cold temperature with as little as one amino acid substitution (Fields and Houseman, 2004; Fields and Somero, 1998; Holland et al., 1997; Johns and Somero, 2004). None of the GPAT isoforms have been characterized in fishes and none have been sequenced in a cold-adapted species.

I investigated the interrelationship between mitochondrial density and mitochondrial membrane phospholipid synthesis in cold-adapted and cold-acclimated fishes and tested the following hypotheses:

(1). GPAT activity is higher in oxidative muscle of cold-acclimated stickleback than warm-acclimated stickleback. Mitochondrial density increases in oxidative muscle, but not in liver tissue, in response to cold acclimation in temperate stickleback, increasing the demand of lipid production, therefore GPAT activity also increases in pectoral muscle, and not liver, in response to cold acclimation in the temperate stickleback. To test this hypothesis, threespine stickleback were acclimated to 8 °C (cold-acclimated) or 20 °C (warm-acclimated) for 11 weeks. Maximum activity of GPAT and transcript levels of the mitochondrial isoforms GPAT1 and GPAT2 were measured in pectoral muscle and liver using quantitative real-time PCR (qRT-PCR).

(2). GPAT1 and GPAT2 mRNA levels are higher in heart ventricles of the icefish *Chaenocephalus aceratus* (-Hb/-Mb) compared to the red-blooded notothenioid *Notothenia coriiceps* (+Hb/+Mb) due to higher mitochondrial densities in hearts of icefishes compared to red-blooded species. To test this hypothesis, transcript levels of GPAT1 and GPAT2 were quantified in liver and ventricle tissue of *N. coriiceps* and *C. aceratus* using qRT-PCR.

(3). Amino acid substitutions in GPAT1 of Antarctic species may increase hydrophobicity and structural flexibility to maintain protein function at cold temperature compared to sub-Antarctic fishes. Amino acid substitutions may also occur in predicted transmembrane domain regions I and II, reflecting the higher proportion of polyunsaturated fatty acids within mitochondrial membranes of Antarctic fishes compared to sub-Antarctic fishes. These hypotheses were tested by sequencing GPAT1 cDNA in hearts of three notothenioid species: Antarctic notothenioids *C. aceratus* and *N. coriiceps* (inhabiting waters at -1.8-2°C), and the sub-Antarctic species, *Eleginops maclovinus*, which inhabits warmer waters around the Argentinian Patagonian shelf and the Pacific coast (at temperatures of 4-10 °C) and diverged prior to the formation of the circumpolar current and isolation of Antarctica.

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CHAPTER 1. Characterization of GPAT1 in Antarctic notothenioid fishes[1]

1.1 ABSTRACT

Hearts of Antarctic hemoglobinless icefishes (suborder Notothenioidei, family Channichthyidae) have higher densities of mitochondria, and mitochondria have higher densities of phospholipids, compared to red-blooded notothenioids. Glycerol-3-phosphate acyltransferase (GPAT) synthesizes lysophosphatidic acid (LPA) from glycerol-3-phosphate and fatty acyl CoA, and is considered the rate-limiting step in phospholipid and triacylglyceride biosynthesis. There are at least three isoforms of GPAT in fish, which are homologous to mammalian GPAT1, GPAT2, and GPAT3. Mammalian GPAT1 and GPAT2 are localized to the outer mitochondrial membrane, whereas GPAT3 is localized to the endoplasmic reticulum membrane. We hypothesized that transcript levels of GPAT1 and/or GPAT2 would be correlated with densities of mitochondrial phospholipids and higher in the icefish *Chaenocephalus aceratus* compared to the red-blooded species *Notothenia coriiceps*. Transcript levels were quantified in hearts and liver of both species. Additionally, GPAT1 cDNA was sequenced in *C. aceratus* and *N. coriiceps* and in the sub-Antarctic notothenioid, *Eleginops maclovinus*, to identify amino acid substitutions that may maintain GPAT1 function at cold temperature. Expression of GPAT1 was highest in liver of *N. coriiceps* but there was no difference in mRNA levels between hearts of *C. aceratus* and *N. coriiceps* despite differences in mitochondrial membrane density. GPAT2 transcripts were undetectable. Together, these data suggest GPAT3 may contribute to the regulation of mitochondrial phospholipid synthesis. GPAT1 amino acid sequences are highly conserved among the three notothenioids (97.9-98.1%). Amino acid substitutions of GPAT1 in the two Antarctic notothenioids compared to the sub-Antarctic species *E. maclovinus* include Ser415Ala, Asp603Glu and Thr648Ala, which are located in the cytosolic region of the protein and may maintain conformational changes crucial to binding and catalysis at chronically cold temperatures.

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1.2 INTRODUCTION

Mitochondrial density increases up to 2-fold in oxidative skeletal muscle of temperate fish species in response to cold acclimation (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Tyler and Sidell, 1984). Elevations in mitochondrial density offset the depressive effects of cold temperature on the catalytic rate of enzymes and increase the diffusion rate of metabolites by minimizing diffusion distances (Sidell, 1998). High mitochondrial densities are also characteristic of oxidative muscles of polar fishes (Johnston et al., 1998). The percent cell volume occupied by mitochondria in oxidative skeletal muscle of moderately active, demersal Antarctic fishes ranges between 29 and 33% compared to 8-13% in Mediterranean species (Johnston et al., 1998). Members of the hemoglobinless Channichthyidae family of Antarctic notothenioids lie at the extreme end of this continuum with mitochondrial densities in heart ventricles ranging between 20% and 37 % and in oxidative pectoral adductor muscle between 39% and 51% (Archer and Johnston, 1991; Feller et al., 1985; Johnston et al., 1998; O'Brien and Sidell, 2000; O'Brien et al., 2003). Surprisingly, high mitochondrial densities in icefish oxidative muscles do not enhance aerobic metabolic capacity, as maximal activities of citrate synthase (CS) and cytochrome *c* oxidase, and mRNA levels of CS are similar between hearts of red- and white blooded notothenioids (Johnston and Harrison, 1985; O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). Rather, high mitochondrial densities likely compensate for the lack of Hb and myoglobin (Mb), the intracellular oxygen-binding protein in icefishes, and enhance oxygen storage and diffusion (Sidell, 1998).

Six of the sixteen species of icefishes lack Mb in cardiac myocytes, and all notothenioids lack Mb in oxidative skeletal muscle (Moylan and Sidell, 2000; Sidell et al., 1997). Mb stores oxygen within cells and enhances oxygen diffusion to mitochondria (Wittenberg, 1970). These functions of Mb appear to be at least partially restored by mitochondrial membranes in oxidative muscles of icefishes (Wittenberg, 1970). Higher mitochondrial densities in oxidative muscles of icefishes are correlated with higher densities of lipids. The two most abundant mitochondrial phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are 1.3- to 1.4- fold higher per g mitochondrial protein in hearts of the icefish *C. aceratus* compared to the red-blooded species *N. coriiceps* (O'Brien and Mueller, 2010). The lipid-rich mitochondria in icefish hearts facilitate oxygen diffusion because oxygen is approximately four times more soluble in

phospholipids than in water (Runswick et al., 1989; Smotkin et al., 1991). Moreover, oxygen concentrates within the hydrophobic core of the phospholipid bilayer, increasing oxygen storage capacity in muscle (Sidell, 1998). The overall increase in mitochondrial phospholipids is likely attributable to an upregulation in the glycerolipid synthesis pathway, yet it is unknown how the synthesis of phospholipids is integrated into mitochondrial biogenesis in either fish or mammals. The enzyme glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the rate-limiting step in phospholipid biosynthesis, may regulate mitochondrial phospholipid density and contribute to differences in mitochondrial densities between red- and white-blooded notothenioids.

GPAT catalyzes the synthesis of lysophosphatidic acid (LPA) from glycerol-3-phosphate (G3P), the backbone of all glycerolipids, and fatty acyl-CoA. LPA is then acylated by 1-acylglycerol-3-phosphate transferase (AGPAT), also known as LPA acyltransferase (LPAAT), to form phosphatidic acid (PA), a precursor to all triacylgylcerols (TAGs) and phospholipids (Coleman and Lee, 2004). In mammals, there are at least four GPAT isoforms (GPAT1-4) differing in their subcellular location, sensitivity to the sulfhydryl reagent N-ethylmaleimide (NEM), and fatty acyl-CoA preference (Gimeno and Cao, 2008; Takeuchi and Reue, 2009; Wendel et al., 2009). Genome sequences of the model fish species *Gasterosteus aculeatus* and *Danio rerio* predict at least three GPAT isoforms in fish, homologous to mammalian GPAT1, GPAT2 and GPAT3. GPAT1 and GPAT2 are mitochondrial isoforms associated with the outer mitochondrial membrane with molecular masses of 92-kDa and 88-kDa, respectively (Coleman and Lee, 2004). GPAT3 and GPAT4 are localized to the endoplasmic reticulum (ER) membrane and have molecular masses of 50 kDa and 52.2 kDa, respectively (Beigneux et al., 2006; Cao et al., 2006). GPAT1, GPAT2 and GPAT3 contain two transmembrane domains, whereas GPAT4 has three (Takeuchi and Reue, 2009). In GPAT 1, 3 and 4, the active site faces the cytosol, whereas in GPAT2, motifs I-III of the active site face the cytosol and motif IV it is located within the first transmembrane domain (Gonzalez-Baro et al., 2001; Nakagawa et al., 2012). GPAT1 is insensitive to NEM and prefers saturated fatty acids to unsaturated fatty acids, whereas GPAT2, GPAT3, and GPAT4 are sensitive to NEM and do not demonstrate a preference for either saturated or unsaturated fatty acyl donors (Gimeno and Cao, 2008; Nagle et al., 2008; Takeuchi and Reue, 2009; Vancura and Haldar, 1992).

Activity and expression of each isoform varies with tissue type. GPAT1 is highly expressed in liver, adipose tissue, oxidative skeletal muscle, heart, brain and kidney (Coleman and Lee, 2004; Lewin et al., 2008; Lewin et al., 2001; Wang et al., 2007). GPAT2 is 50-fold more abundant in mouse testis than other tissues, including liver and heart, with levels higher in liver than heart (Wang et al., 2007). GPAT2 expression in mice is higher in heart and liver than GPAT1 (Wang et al., 2007). Activity of GPAT1 comprises up to 30% of total GPAT activity and 43% of mitochondrial GPAT activity in the hearts of mice (Coleman and Lee, 2004; Lewin et al., 2008). Both GPAT3 and GPAT4 mRNA are abundant in mouse adipose tissue, together accounting for ~50% of total GPAT activity in adipose with GPAT3 being higher in white adipose tissue and GPAT 4 being higher in brown adipose tissue (Shan et al., 2010).

To date, the functional differences between the mitochondrial and microsomal GPAT isoforms are largely unknown. Experiments with GPAT1 knockout mice show decreased liver and plasma TAGs and increased fatty acid oxidation, and mice over expressing GPAT1 show an increase in TAG content and a decrease in fatty acid oxidation in hepatocytes, supporting the role of GPAT1 in regulating TAG synthesis (Hammond et al., 2002; Igal et al., 2001; Lewin et al., 2005). GPAT1 may regulate fatty acid composition of phospholipids in liver, as PE and PC contain 21% less palmitate (the preferred fatty acid substrate of GPAT1) in the *sn*-1 position in GPAT1-null mice (Hammond et al., 2002). Additionally, GPAT1-null-mice also have altered heart phospholipid compositions as they contain lower amounts of palmitoyl-CoA in PE and PC, higher amounts of stearic acid and oleic acid, and significantly more amounts of arachidonic acid (Lewin et al., 2008). GPAT2 has been shown to play a minor role in initiating TAG synthesis; mRNA abundance is not altered in response to diet and does not compensate for decreases in GPAT1 activity (Wang et al., 2007). Recent studies have shown that GPAT mitochondrial isoforms are essential for mitochondrial fusion and regulating mitochondrial dynamics and morphology in *Caenorhabditis elegans* and mammals (Ohba et al., 2013). The contribution of mitochondrial GPAT to mitochondrial morphology in fishes has not been studied.

We hypothesized that if GPAT1 and GPAT2 are involved in synthesizing mitochondrial membranes, that their expression would be higher in heart ventricles of the icefish *C. aceratus* (-Hb/-Mb) compared to the red-blooded species *N. coriiceps* (+Hb/+Mb). We also predicted that expression of GPAT1 would be higher than GPAT2 in heart and lower than

GPAT2 in liver of both *C. aceratus* and *N. coriiceps*, consistent with the expression pattern observed in mammals (Wang et al., 2007). Transcript levels of GPAT1 and GPAT2 were quantified in liver and ventricle tissue of *N. coriiceps* and *C. aceratus* using real-time quantitative PCR. Additionally, GPAT1 cDNA was sequenced in hearts of three notothenioid species: *C. aceratus, N. coriiceps,* and the sub-Antarctic species *Eleginops maclovinus*, which inhabits warmer waters around the Argentinian Patagonian shelf and the Pacific coast (4-10 °C) and diverged prior to the isolation of Antarctica and formation of the circumpolar current (Eastman, 1993), to provide insight to the evolution of a mitochondrial transmembrane protein in fishes inhabiting a chronically cold environment.

1.3 MATERIAL AND METHODS

1.3.1 *Tissue Collection*

N. coriiceps (Richardson, 1844) and *C. aceratus* (Lönnberg, 1905) were captured in Dallmann Bay (64°10' S, 62°35'W) and off the south western shore of Low Island (63°24'S, 62°10'W) using an otter trawl deployed from the ARSV *Laurence M. Gould* during the austral autumn of 2007, 2009, 2011, and 2013. Animals were maintained in circulating seawater tanks on board the ship then transferred to circulating seawater tanks at the U.S. Antarctic Research Station, Palmer Station on Anvers Island where they were maintained at 0 ± 0.5° C. Fish were euthanized by either a sharp blow to the head or by an overdose of tricaine methane sulfonate (MS-222) (1:7500 in seawater) followed by spinal cord transection. Heart ventricles and livers were excised immediately, frozen in liquid nitrogen and stored at -80° C. Heart ventricles of the sub-Antarctic notothenioid species, *E. maclovinus* (Cuvier), caught in 2004, were generously donated by Dr. Bruce Sidell (University of Maine). All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee.

1.3.2 *RNA isolation and cDNA synthesis*

Total RNA was extracted from heart ventricles and liver tissues using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) except that RNA was treated with DNase I twice, once for 25 and again for 20 min. Concentration and purity of RNA were determined spectrophotometrically with a Nanodrop ND-1000 spectrophotometer (ThermoScientific Fisher,

Pittsburgh, PA, USA). All samples had 260 nm-to-230 nm ratios of 1.8 - 2.3 and 260 nm-to-280 nm ratios of 2.0 - 2.2. Integrity of RNA was determined by separating RNA on a 2% agarose gel stained with ethidium bromide. RNA was stored at -80° C until further use. First-strand complimentary DNA (cDNA) was synthesized using Taqman reverse transcription reagents (Applied Biosystems). Each 50 µl reaction volume contained 1 mg RNA, 1 X RT buffer, 3 mM $MgCl_2$, 2 mM dNTPs, 2.5 µM random hexamers, 20U RNase inhibitor, and 37.5U reverse transcriptase. cDNA was stored at -20°C.

1.3.3 *Housekeeping gene analysis*

Transcript levels of 18S ribosomal RNA (18S), elongation factor 1-A (EF1-α), and TATA box binding protein (TBP) were measured in ventricle and liver tissue using qRT-PCR in six individuals of *N. coriiceps* and *C. aceratus* to identify a suitable housekeeping gene (HKG). The final volume of reaction mixtures was 20 µl and contained 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 300 nM forward and reverse primers and 5 ng of cDNA, except that 1 ng cDNA was used as template in 18S reactions. Results were analyzed using the Excel-based software tool BestKeeper (Pfaffl et al., 2004). BestKeeper does not account for differences in gene expression due to differences in experimental conditions; therefore an ANOVA was also performed to identify significant differences in HKG expression between species and tissues.

1.3.4 *Measuring GPAT1 and GPAT2 transcript levels*

Conserved regions of the full-length GPAT1 cDNA sequences and partial GPAT2 cDNA sequences from *N.coriiceps* and *C. aceratus* were used to design gene-specific primers (GSP) using Primer Express version 2.0 (Applied Biosystems) (Table 1.1). For all genes, either the forward or reverse primer was designed over a splice site to ensure genomic DNA was not amplified. Quantitative real-time PCR (qRT-PCR) was carried out using an ABI PRISM 7900HT. The final reaction volume of 20 µl contained 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 300 nM forward and reverse primers, and 5 ng of cDNA. Optimum primer concentration was determined by measuring GPAT1 mRNA levels using primer concentrations of 200, 300, and 400 nM. The cDNA from all individuals and all tissues was pooled, serially diluted, and measured in triplicate to create a standard curve to

determine reaction efficiency. Target genes were measured in triplicate in 6 individuals per tissue and species collected in 2013. Transcript levels of GPAT1 and GPAT2 genes were normalized to transcript levels of TATA box binding protein (TBP) using the comparative critical threshold (ΔΔCt) method (Livak and Schmittgen, 2001). Expression levels of target genes were expressed relative to levels in *N. coriiceps* ventricle. Two controls were run, one lacking cDNA and the other containing cDNA synthesized without reverse transcriptase to ensure that contaminating or genomic DNA was not amplified. Melting curve analysis was conducted to verify that only one product was amplified with each primer set.

1.3.5 *Sequencing GPAT1 and GPAT2 cDNA*

Degenerate primers were designed based on homologous amino acid sequences of GPAT1 in four fish species (*Gasterosteus aculatus, Dicentrarhus labrax, Oreochromis niloticus and Takifugu rubripies*) using CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/codehop.html), and used to amplify a 502 bp segment of GPAT1 from cDNA from *N. coriiceps* ventricle (Table 1.2). PCR was carried out with 3 µl of cDNA, 1 X PCR buffer, 2 mM $MgCl_2$, 0.4 mM dNTP's, 1 µM forward and reverse primers, and 2.5U Taq polymerase (Promega) using a touchdown protocol with an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). A forward gene specific primer (GSP) for GPAT1 was then designed based on the GPAT1 sequence in *N. coriiceps* and paired with a degenerate reverse primer, designed as described above, to amplify a 1250 bp region using the PCR conditions described above and annealing temperatures between 63° C and 60° C (Table 1.2). For GPAT2, degenerate primers were designed as described above to amplify a 1350 bp segment in *N. coriiceps* and *C. aceratus* (Fig. 1.3 and Tables 1.2 and 1.3). 5' and 3' cDNA ends in GPAT1 were amplified using the SMARTer cDNA Amplification kit (Clontech, Mountain View, CA USA) and gene-specific primers (Table 1.2), except for the 3' end of GPAT1 in *E. maclovinus*, for which gene-specific primers were used (Table 1.2). PCR products were size-separated on 2% agarose gels and stained with ethidium bromide. Products of expected length were excised, isolated using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), and cloned into *Escherichia coli* using the pCR 2.1®-TOPO vector and TOP10 One Shot® chemically competent cells (TOPO TA Cloning, Invitrogen, Carlsbad, CA, USA). Transformed

cells were identified by blue-white screening on LB-agar plates supplemented treated with 25 mg ml^{-} ampicillin and supplemented with 64 μg ml^{-} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). Plasmids were isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Valenica, CA, USA), and used as templates for Sanger sequencing reactions using reagents and instructions from the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reaction products were purified with Centri-Sep columns packed with Sephadex G-50 gel (Sigma-Aldrich, St. Louis, MO, USA), and sequenced with M13 primers on an ABI PRISM 3100 instrument (Table 1.2). Full-length cDNA was sequenced in two individuals of both *N. coriiceps* and *C. aceratus* and one individual for *E. maclovinus*. Sequence identity was determined by searching the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov) for homologous sequences.

Consensus sequences for each species were obtained by the plurality criteria using Sequencher (version 5.1; Gene Codes Corporation). Nucleotide and amino acid alignments were performed in MEGA (version 6.06) using the ClustalW algorithm.

GPAT1 cDNA sequences from the three notothenioids along with cDNA sequences of 8 fish species (*Gasteosteus. aculeatus, Takifugu rubripes, Esox Lucius, Maylandia. zebra, Oreochromis. niloticus, Pundamilia nyererei, Cynoglossus semilaevis*, and *Gadus. morhua*) obtained from GenBank were aligned with the ClustalW algorithm in MEGA. Fishes were grouped as follows: notothenioids, cichlids (*M. zebra, O. niloticus*, and *P. nyererei*), and all fishes (N=11). Alignments were then analyzed for rates of nonsynonymous (dN) and synonymous (dS) substitutions to assess the strength and direction of selective evolutionary pressures via HyPhy in MEGA (version 6.06) using a maximal likelihood analysis.

1.3.6 *Statistical analysis*

All statistical analyses were conducted in R 3.0.1 (R Core Development Team 2013, Vienna, Austria) with significance set at $\alpha < 0.05$. Results are presented as means ± s.e.m. A two-way ANOVA followed by a *post-hoc* Tukey's honestly significant difference (HSD) test was used to determine significant differences in the expression of HKGs and mRNA levels of GPAT1 in ventricle and liver of *N. coriiceps* and *C. aceratus*. Critical threshold values were log

transformed to meet assumptions of equal variance and normality, as determined by Levene's Test for Homogeniety of Variance and a Shapiro-Wilks test.

1.4 RESULTS

1.4.1 *Housekeeping gene analysis*

Expression levels of three potential HKGs were analyzed in heart ventricle and liver of *N. coriiceps* and *C. aceratus* using BestKeeper (Pfaffl et al., 2004). These included 18S ribosomal RNA (18S rRNA), elongation factor 1-A (EF1-α), and TATA box binding protein (TBP). Transcript levels of all HKGs had standard deviations < 1.0 and were significantly correlated with the Bestkeeper index with the exception of 18S in liver (Table 1.4). When tissues were pooled for each HKG, TBP was not correlated with the Bestkeeper index. However, the two-way ANOVA determined that there was no significant difference in TBP expression level among species and tissues but Ct values for 18S and EF1-α were significantly different among tissues and species and therefore these two genes were eliminated, and transcript levels of GPAT1 and GPAT2 were normalized to TBP (Table 1.4).

1.4.2 *GPAT1 and GPAT2 transcript levels*

There were no significant differences in levels of GPAT1 mRNA between *N. coriiceps* and *C. aceratus* in either heart or liver tissue ($P > 0.05$) (Fig. 1.1). However, GPAT1 transcripts and were 5–fold higher in liver compared to ventricle of *N. coriiceps* (Fig. 1.1). GPAT1 mRNA levels did not differ between liver and heart in *C. aceratus*, although they tended to be higher in liver ($P = 0.11$). GPAT2 transcript levels were undetectable in heart ventricle and liver of both species.

1.4.3 *GPAT1 cDNA sequence*

GPAT1 has an open-reading frame of 2484 base pairs in *N. coriiceps* and 2481 base pairs in *C. aceratus* and *E. maclovinus*, corresponding to a coding region of 827 amino acids in *N. coriiceps* and 826 amino acids in *C. aceratus* and *E. maclovinus* (Fig. 1.2). The predicted amino acid sequence of GPAT1 in notothenioids contains two transmembrane domains and four motifs within the active site. The cDNA sequences between the two Antarctic species share

98.6% identity while the two Antarctic and the sub-Antarctic notothenioid *E. maclovinus* share 96-96.1% identity. Amino acid sequences are 98.4% identical between the two Antarctic species, 97.9% between *C. aceratus* and *E. maclovinus* and 98.1% between *N.coriiceps* and *E. maclovinus*. Overall there were ten sites where amino acids in the two Antarctic species differed from those of *E. maclovinus,* however after comparing GPAT1 amino acid sequences of temperate and tropical fishes as well as higher vertebrates, only three substitutions appear to be unique to the two Antarctic notothenioids; Ser415Ala, Asp603Glu and Thr648Ala (Fig. 1.4). At all three positions *E. maclovinus* substitutions mirror those of the temperate species *G. aculeatus* and at position 648 the substitution in *E. maclovinus* mirrors that of not only *G. aculeatus* but also those of all other temperate and tropical fishes used in this study (Fig 1.4).

Active site motifs and transmembrane domains are highly conserved between Antarctic and sub-Antarctic notothenioids. The putative GPAT1 active site in notothenioids is located between amino acids 225-350. All three notothenioid species show a 100% identity in motifs I-IV (Table 1.5). Transmembrane domains I (amino acids 466-488) and II (amino acids 572-589) are 100% identitical among the three notothenioid species (Table 1.5A and 1.6B).

Negative, or purifying, selection is the predominant evolutionary force acting upon GPAT1 in the selected fish species (Fig 1.4). Evolutionary forces acting on GPAT1 were quantified by the dN/dS ratio, which is the ratio of the rate of substitutions at silent sites (dS), which are presumed neutral, to the rate of substitutions at non-silent sites (dN), which possibly experience selection (Kryazhimskiy and Plotkin, 2008). A low ratio (dN/dS <1) indicates strong purifying (''stabilizing'') selection, whereas a high ratio (dN/dS >1) indicates selection for diversification (''positive selection'') and a ratio of one indicates neutral, or no selection (Muse and Gaut, 1994; Rocha et al., 2006). There were 11 dN/dS ratio values >1 in the analysis including all 11 fish species, indicating positive selection at those particular sites however the overall dN/dS mean is -2.67 (Table 1.7). Neither notothenioid nor cichlid groups had dN/dS ratios >1 for any sites and the overall dN/dS means were -0.162 and -0.04, respectively.

1.5 DISCUSSION

Contrary to our expectations, GPAT1 mRNA levels were not higher in the heart ventricle of *C. aceratus* compared to *N. coriiceps*, and GPAT2 transcripts were undetectable, suggesting GPAT3 may be the rate-limiting step governing the synthesis of mitochondrial phospholipids. Additionally, amino acid sequences of GPAT1 between Antarctic and sub-Antarctic notothenioids reflect their phylogenetic relatedness and are highly conserved, sharing 97.9-98.1% identity with three sites potentially reflecting cold-adaptation in Antarctic notothenioids: Ser415Ala, Asp603Glu and Thr648Ala.

Although hearts of the icefish *C. aceratus* have 2.3 fold higher mitochondrial densities compared to closely related red-blooded species (O'Brien and Sidell, 2000), transcript levels of the putative rate-limiting enzyme in glycerolipid synthesis, GPAT1, were similar between heart ventricles of *C. aceratus* and *N. coriiceps*. These results were contrary to our expectations because mitochondrial phospholipids PE and PC are 1.3 to 1.4-fold higher per mg mitochondrial protein in hearts of *C. aceratus* compared to *N. coriiceps* (O'Brien and Mueller, 2010). These results suggest that GPAT1 is not the rate-limiting step in the synthesis of mitochondrial phospholipids. Alternatively, GPAT1 mRNA levels may not reflect protein levels. Consistent with this, in rat, mitochondrial GPAT1 and GPAT2 mRNA levels were 5-fold greater in liver than heart, yet GPAT protein levels were 5-fold greater in heart than liver (Lewin et al., 2001). This discrepancy could be due to the slow rate of protein turnover in heart (Lewin et al., 2001). A possible explanation for the lack of difference in transcript levels of GPAT1 between red- and white-blooded species is that GPAT3, rather than, or in addition to GPAT1, regulates mitochondrial phospholipid biosynthesis.

Transcript levels of GPAT1 may be more closely correlated with the synthesis of TAGs than membrane phospholipids. While the expression of GPAT1 is similar between hearts of red- and white-blooded notothenioids, expression was 5-fold higher in liver of *N. coriiceps* compared to ventricle tissue in *N. coriiceps*. This expression pattern is consistent with GPAT1 levels in heart and liver of mammals, where GPAT1 mRNA levels are 2-fold higher in rat liver than heart (Wang et al., 2007). TAGs are primarily synthesized in the liver and then transported to muscle

to be used as an energy source, and to adipocytes for energy storage. Therefore it is likely that enzymes associated with TAG synthesis would be greater in liver than cardiac muscle of fishes.

GPAT2 transcript levels were undetectable in liver and heart of *N. coriiceps* and *C. aceratus*. This was unexpected because GPAT2 mRNA levels are higher than GPAT1 in mammalian hearts and liver, yet we were unable to detect transcripts of GPAT2 in either tissue of red- and white-blooded notothenioids (Wang et al., 2007). This suggests GPAT2 may not be as ubiquitous in fish as it is in mammals.

GPAT1 amino acid sequences are highly conserved (97.9-98.1%) between Antarctic and sub-Antarctic species. The lower percent identity between Antarctic and sub-Antarctic species compared to percentages between the two Antarctic species (98.4%) likely reflects the divergence of *E. maclovinus* from the other notothenioids approximately 22-23 million years ago (Bargelloni and Lecointre, 1998). The higher degree of similarity between *E. maclovinus* and *N. coriiceps* (98.1%) reflects their closer phylogenetic relationship compared to *E. maclovinus and C. aceratus* (97.9%), as the icefish are predicted to have diverged from the notothenioid lineage between 5.5 and 2 million years ago (Bargelloni et al., 2000; Eastman, 1993).

The four motifs comprising the active site region of GPAT1 are 100% conserved among the three notothenioid species, but differ from tropical fishes. Studies comparing cold-adapted and non-cold-adapted enzyme orthologs have shown that active sites are highly conserved (Crawford et al., 1989; Fields and Somero, 1998; Johns and Somero, 2004). For GPAT1, active site motifs I, II and IV were 100% identical between notothenioids and temperate and tropical fishes but motif III, one of the motifs associated with both G3P binding and catalysis, contains the substitution Phe307Tyr in tropical fishes, which may impact catalytic rate due to differences in hydrophobicity of the R groups between Tyr and Phe. Tyr is less hydrophobic and could be either buried within the protein or partially exposed (Dircks et al., 1999; Lewin et al., 1999). This substitution is interesting because the Phe (313), along with Gly (316) and Arg (318), in motif III of GPAT1 of mammals are considered crucial for catalysis (Coleman and Lee, 2004; Gonzalez-Baro et al., 2001; Wang et al., 2007). The three notothenioid species along with temperate fishes *G. aculeatus* and *T. rubripes* have the Tyr substitution in motif III while the tropical fishes, as well as higher vertebrates, have Phe in their corresponding positions.

Our study found no differences in the transmembrane regions of GPAT1 between Antarctic and sub-Antarctic notothenioids, but there were differences between notothenioids and temperate and tropical fish species. We anticipated that sequences of GPAT1 transmembrane domains would reflect differences in membrane phospholipid composition between Antarctic and sub-Antarctic species. The percentage of unsaturated fatty acids in membrane phospholipids is inversely correlated with body temperature, therefore Antarctic fishes have membranes rich in PUFAs (Logue et al., 2000). There are, however, a couple of amino acid substitutions in the two transmembrane domains between notothenioids and temperate and/or tropical fishes: Val482Ile in transmembrane domain I and Val585Ile in transmembrane domain II. Both Ile and Val are β carbon branched amino acids that can create bulkiness near the protein backbone, however Ile is slightly more bulky because of the additional methyl group compared to Val. The presence of Ile, rather than Val, may facilitate insertion of GPAT into a highly unsaturated, and therefore loosely packed, membrane as in the case of cold-bodied fishes.

Amino acid substitutions outside of the active site and transmembrane domains of GPAT1 that differ between the two Antarctic notothenioids and sub-Antarctic notothenioid *E. maclovinus* include: Ser415Ala, Asp603Glu and Thr648Ala. Although both Ala and Ser are small amino acids, the Ala in Antarctic notothenioids at position 415 is a nonpolar amino acid while the hydroxyl group in polar Ser is fairly reactive and can easily form hydrogen bonds and increase protein stability. The same differences in hydrophobicity, or polarity, are true for the substitution Thr648Ala in that Ala is nonpolar and Thr is polar but Thr is a β carbon branched amino acid and Ala is not. As previously mentioned, β carbon branched amino acids create bulkiness and therefore restriction in conformations of the main chain, increasing rigidity and stability of the protein. These Ala substitutions in the Antarctic notothenioids, which likely increase flexibility and decrease activation energy, are ones we would expect to see in cold-adapted proteins (D'Amico et al., 2002). However the Glu in Antarctic notothenioids at position 603 has a longer side chain than Asp and is less rigid within protein structures.

Sequence identity of GPAT1 between fish and birds and mammals ranges from 62-64%. Notably, fish have a five amino acid length sequence that is absent in mammals and birds. It is located downstream from the second transmembrane domain (amino acids 604-608 and 603-607 in notothenioids) and within the cytosolic-facing C-terminal domain, where it may interact with

substrates or play a role in conformational changes during catalysis (Pellon-Maison et al., 2006). In mammals, the entire C-terminal domain (amino acids 594-828) of GPAT1 is necessary for activity and interacts with the N-terminal domain in the cytosol (Pellon-Maison et al., 2006). Future research might investigate the function of this amino acid sequence in fishes.

Purifying selection was determined to be the predominant evolutionary force acting upon GPAT1 among the three notothenioids. Purifying, or negative selection, decreases variability and reduces the frequency of deleterious alleles. In contrast, variants that increase the fitness of an individual in its environment may increase in frequency as a result of positive selection (Bamshad and Wooding, 2003; Lawrie et al., 2013). While some differences in amino acids between the two Antarctic species and *E. maclovinus* (3 positions) may have been caused by positive selection, the overall dN/dS of -0.162 indicates that GPAT1 has undergone negative selective pressure to the degree that the gene remains highly conserved between Antarctic and sub-Antarctic species, however dN/dS does not address functional changes which can only be determined via functional studies such as site-directed mutagenesis.

Purifying selection is also the predominant evolutionary force acting on GPAT1 among the eleven fish species we selected to analyze. Overall, evolutionary pressures have occurred to conserve the ancestral state of GPAT1 among the Antarctic, sub-Antarctic, temperate and tropical fish orthologs (dN/dS mean -2.67), indicating selection for maintaining GPAT1 function. Genes enriched in strongly constrained synonymous sites tend to be particularly functionally important (Lawrie et al., 2013). For example, among higher eukaryotic species, histones genes, such as those coding for H4 proteins, are highly conserved and show almost identical amino acid sequences within and between species with purifying selection being the major force at the protein level (Piontkivska et al., 2002). Additionally, ribosomal RNA genes as well as other universally conserved nucleotide sequences in genes encoding particular classes of transfer RNAs are considered among the most conserved DNA sequences (Isenbarger et al., 2008). Given that GPAT is considered the rate-limiting enzyme in glycerolipid synthesis, it is conceivable that it would be highly conserved across species, supporting its important function in metabolism and membrane synthesis. There were, however, eleven sites among the eleven fishes where GPAT1 appears to be under positive selection, with dN/dS >1. Two are located within the active site

(position 342 and 343), however neither are part of an actual motif, but rather are positioned between motifs III and IV. Another dN/dS analyses of *N. coriiceps* comparing over 5,000 orthologs found that the average dN/dS ratio of *N. coriiceps* (0.133) was significantly higher than that of the fishes *G. aculeatus, Danio rerio, Tetradon nigroviridis, T. rubripes* and *G. Morhua* (0.050 to 0.115), likely due to the high selective pressure caused by the harsh Antarctic environment (Shin et al., 2014). Similarly, The dN/dS of GPAT1 in notothenioid group was -0.162 compared to -0.04 in GPAT1 in the cichlid group, indicating more selective pressure in the notothenioids, possibly due to the lower average habitat temperature of notothenioids compared to cichlids.

1.6 ACKNOWLEDGEMENTS

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1.7 FIGURES

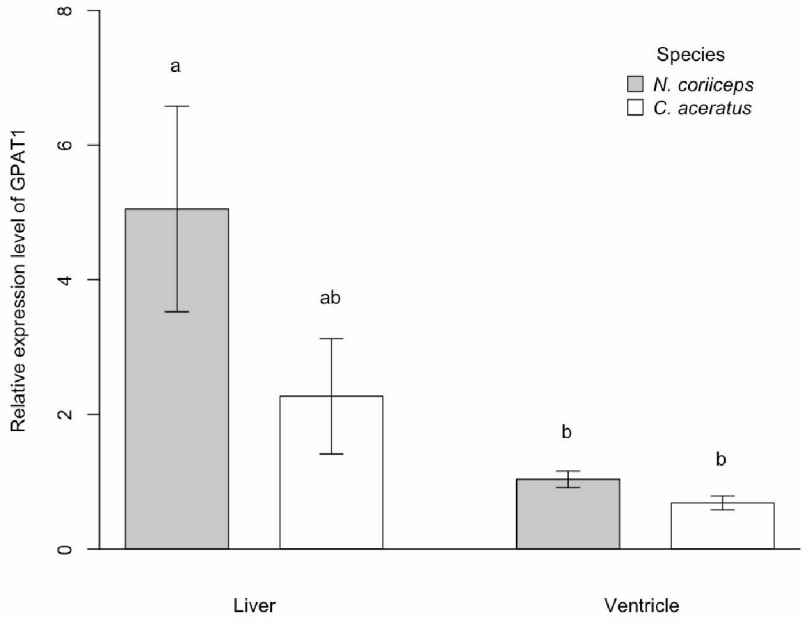
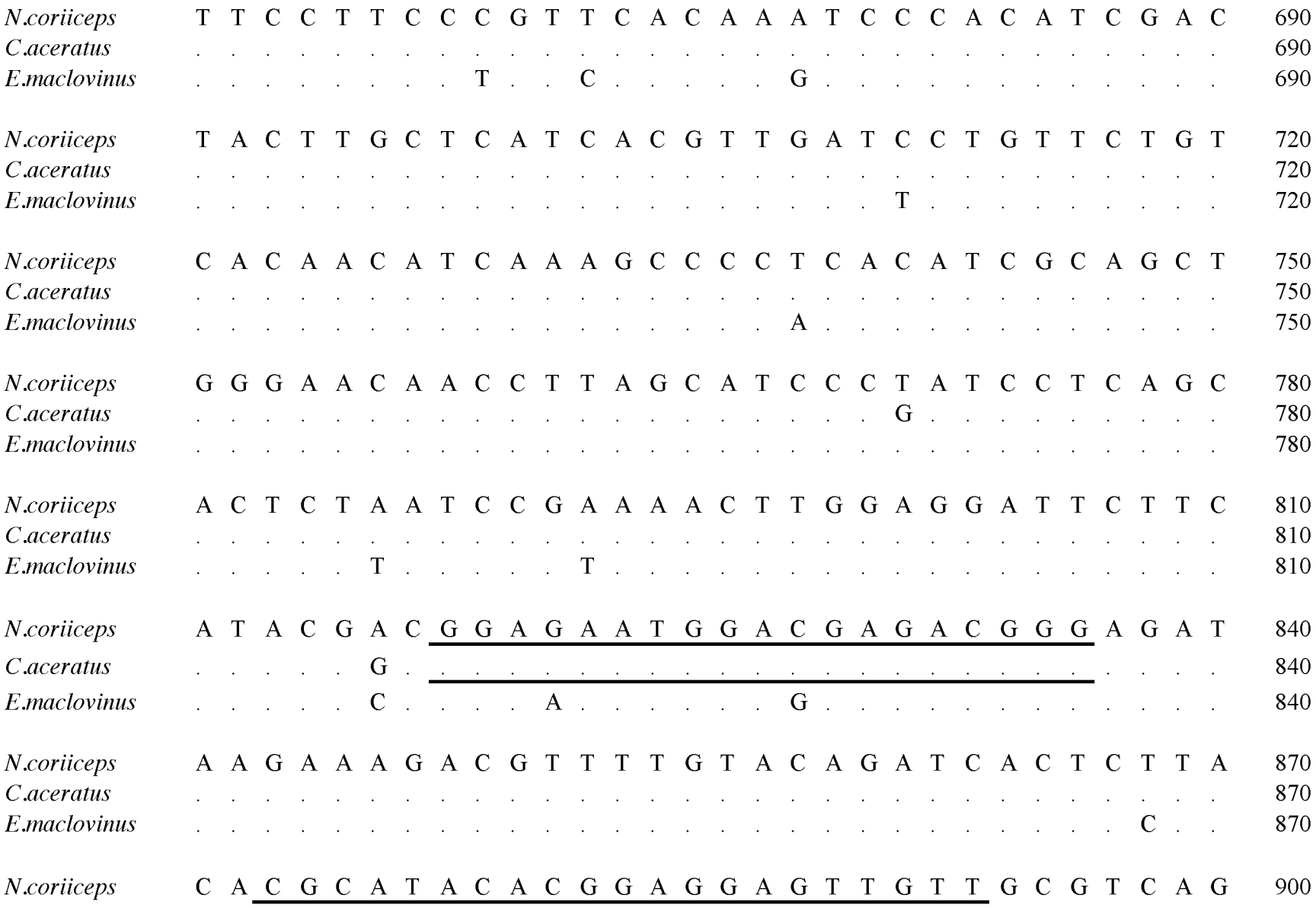


Figure 1.1. GPAT1 mRNA levels in heart and liver of notothenioids. Relative expression levels of GPAT1in ventricle and liver tissue of the red-blooded species *N. coriiceps* and icefish species *C. aceratus*. Transcript levels were normalized to the HKG TBP. Significant differences are indicated by different letters. (N=6).

N.coriiceps	A	T	G	G	C	G	C	T	G	T	C	A	G	A	C	G	G	C	T	T	G	T	T	G	C	T	G	C	A	G	30
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	30
E.maclovinus	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	30
N.coriiceps	G	T	C	A	A	C	A	A	T	G	G	A	G	A	G	C	A	G	T	G	G	A	C	T	A	A	C	C	G	C	60
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	60
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	60
N.coriiceps	T	G	G	A	A	A	C	A	T	C	C	C	A	A	C	G	A	T	G	A	C	T	C	G	G	A	T	C	G	C	90
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	90
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	90
N.coriiceps	A	G	C	A	C	C	A	G	C	C	C	C	T	C	T	G	T	C	C	T	G	C	G	C	T	G	C	G	T	C	120
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	120
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	120
N.coriiceps	G	C	C	A	G	C	A	C	G	T	G	G	A	A	G	G	A	G	G	G	C	C	T	G	C	T	G	A	G	C	150
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	150
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	150
N.coriiceps	A	G	A	A	A	G	A	G	G	C	C	C	T	T	T	G	T	G	G	G	C	C	G	C	T	G	C	T	G	T	180
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	180
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	180
N.coriiceps	C	A	C	T	C	C	T	G	C	A	C	T	C	C	A	C	A	G	A	G	C	T	G	G	G	A	G	A	A	A	210
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	210
E.maclovinus	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	210

N.coriiceps	C	T	G	T	T	C	A	A	C	C	C	C	A	G	T	A	T	C	C	C	A	T	C	T	C	T	G	G	G	A	240
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	240
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	240
N.coriiceps	C	T	G	C	G	C	A	A	T	G	T	C	A	T	C	T	T	C	A	T	T	A	A	T	G	A	G	A	C	C	270
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	270
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	270
N.coriiceps	C	A	C	A	C	C	A	G	G	C	A	G	C	G	G	G	G	C	T	G	G	C	T	G	G	C	G	C	G	C	300
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	300
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	300
N.coriiceps	A	G	G	C	T	G	A	G	T	T	A	T	G	T	G	T	T	G	T	T	T	G	T	C	A	T	G	G	A	G	330
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	330
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	330
N.coriiceps	A	G	A	G	A	T	G	T	C	A	A	C	A	A	G	G	A	C	A	T	G	T	T	C	A	C	C	A	G	G	360
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	360
E.maclovinus	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	360
N.coriiceps	A	A	C	G	T	A	G	T	G	G	A	C	A	A	C	G	T	G	C	T	C	A	A	C	A	A	C	A	G	C	390
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	390
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	390
N.coriiceps	A	G	G	G	T	G	G	A	G	A	G	T	G	C	G	A	T	T	G	C	G	A	A	T	G	T	A	G	C	C	420
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	420
E.maclovinus	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	420
N.coriiceps	G	C	G	G	A	T	G	T	G	G	A	C	G	C	T	G	C	A	G	G	A	A	C	A	C	A	A	C	C	T	450
C.aceratus	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	450

E.maclovinus	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	450
N.coriiceps	G	G	C	C	A	G	G	A	G	C	A	C	A	A	A	G	C	G	G	T	C	A	G	A	A	A	A	G	T	G	480
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	480
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	480
N.coriiceps	A	A	G	C	A	G	A	A	A	G	C	C	C	G	G	G	C	C	T	T	C	C	T	T	C	A	G	G	A	G	510
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	510
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	510
N.coriiceps	A	T	G	G	T	G	G	C	C	A	A	C	A	T	T	T	C	A	C	C	G	G	C	C	T	T	C	A	T	C	540
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	540
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	540
N.coriiceps	A	G	G	C	T	G	A	C	C	G	G	C	T	G	G	G	T	C	C	T	C	C	T	G	A	G	G	C	T	C	570
C.aceratus	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	570
E.maclovinus	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	570
N.coriiceps	T	T	T	A	A	T	G	G	T	T	T	C	T	T	C	T	G	G	A	G	C	A	T	C	C	A	G	A	T	C	600
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	600
N.coriiceps	C	A	C	A	A	G	G	G	T	C	A	G	C	T	G	G	A	A	A	T	A	G	T	C	A	A	A	A	A	A	630
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	630
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	630
N.coriiceps	G	C	T	G	C	G	A	C	A	G	A	G	C	A	A	A	A	T	G	T	G	C	C	C	A	T	G	G	T	C	660
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	660
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	660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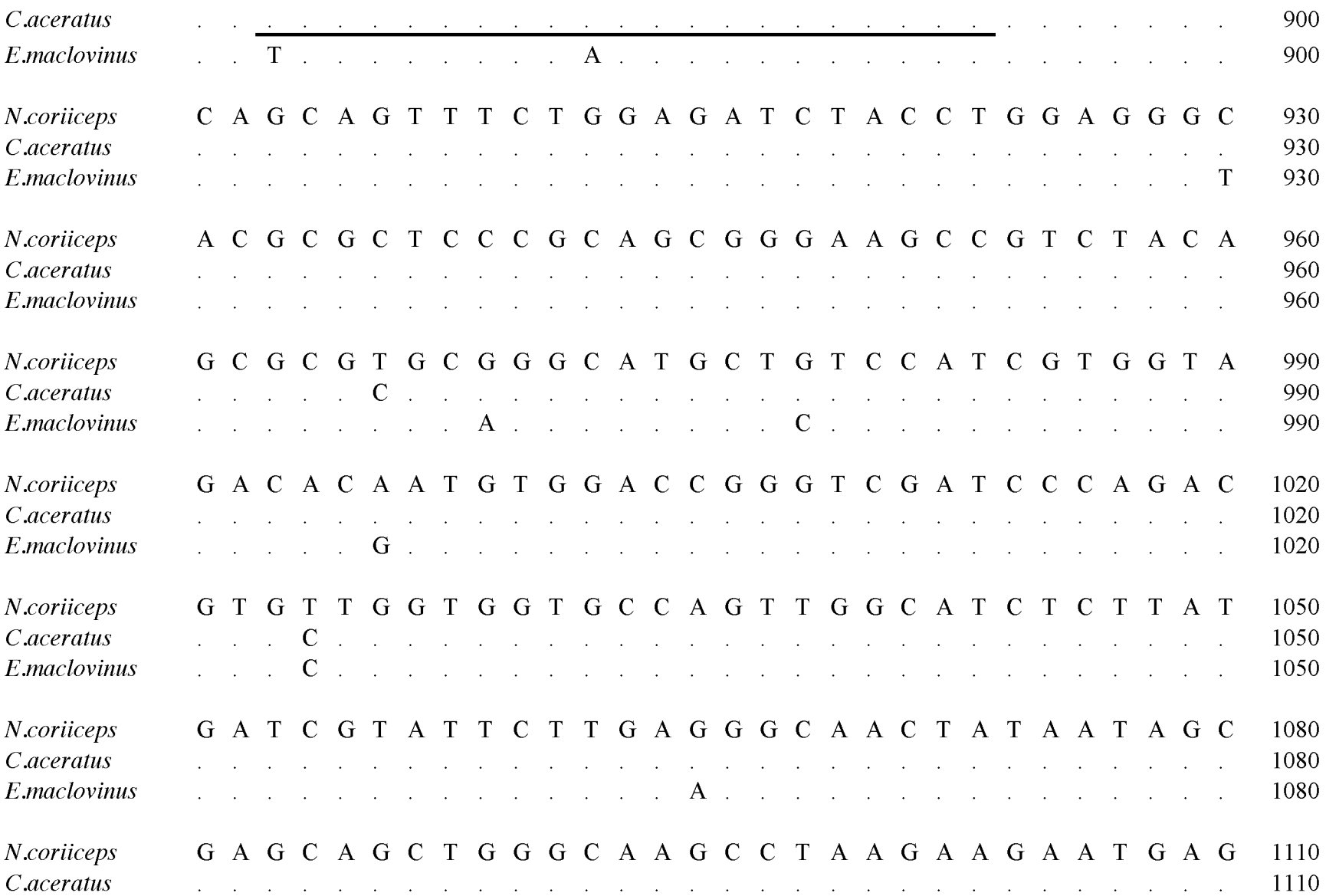
N.coriiceps T A C T T G C T C A T C A C G T T G A T C C T G T T C T G T 720
C.aceratus 720
E.maclovinus . . . . T 720
N.coriiceps C A C A A C A T C A A A G C C C C T C A C A T C G C A G C T 750
C.aceratus 750
E.maclovinus . . . . A 750
N.coriiceps G G G A A C A A C C T T A G C A T C C C T A T C C T C A G C 780
C.aceratus . . . . G 780
E.maclovinus 780
N.coriiceps A C T C T A A T C C G A A A A C T T G G A G G A T T C T T C 810
C.aceratus 810
E.maclovinus . . . . . T . . . . . T 810
N.coriiceps A T A C G A C G G A G A A T G G A C G A G A C G G G A G A T 840
C.aceratus . . . . . G 840
E.maclovinus . . . . . C . . . . A . . . . . G 840
N.coriiceps A A G A A A G A C G T T T T G T A C A G A T C A C T C T T A 870
C.aceratus 870
E.maclovinus . . . . . C . . 870
N.coriiceps C A C G C A T A C A C G G A G G A G T T G T T G C G T C A G 900


C.aceratus 900
E.maclovinus . . T . . . . . A 900
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C.aceratus 930
E.maclovinus . . . . . T 930
N.coriiceps A C G C G C T C C C G C A G C G G G A A G C C G T C T A C A 960
C.aceratus 960
E.maclovinus 960
N.coriiceps G C G C G T G C G G G C A T G C T G T C C A T C G T G G T A 990
C.aceratus . . . . . C 990
E.maclovinus . . . . . A . . . . . C 990
N.coriiceps G A C A C A A T G T G G A C C G G G T C G A T C C C A G A C 1020
C.aceratus 1020
E.maclovinus . . . . . G 1020
N.coriiceps G T G T T G G T G G T G C C A G T T G G C A T C T C T T A T 1050
C.aceratus . . . C 1050
E.maclovinus . . . C 1050
N.coriiceps G A T C G T A T T C T T G A G G G C A A C T A T A A T A G C 1080
C.aceratus 1080
E.maclovinus . . . . . A 1080
N.coriiceps G A G C A G C T G G G C A A G C C T A A G A A G A A T G A G 1110
C.aceratus 1110

E.maclovinus	. . . .	1110
N.coriiceps	A G T T T G T G G G G G A T T G C A T G T G G A G T G T T T	1140
C.aceratus	. . . . A	1140
E.maclovinus	. . . . A C	1140
N.coriiceps	A G G A T G C T G A G G A A G A A C T A C G G T T G T G T T	1170
C.aceratus	. . . .	1170
E.maclovinus	. . . . C . . .	1170
N.coriiceps	C G A G T T G A C T T C A A T C A G C C C T T C T C C T T A	1200
C.aceratus	. . . .	1200
E.maclovinus	. . . . C	1200
N.coriiceps	A A G G A G T A C C T G G A T T C C C A G A G A A A C C G C	1230
C.aceratus	. . . .	1230
E.maclovinus	. . . .	1230
N.coriiceps	C A T A T T C C T C C A G C A G T G T C T C T G G A G C A C	1260
C.aceratus	. . . . A	1260
E.maclovinus	. . . . T	1260
N.coriiceps	A C C T T G A T G C C C A T C A T C A T T T C T G C A C A A	1290
C.aceratus	. . . . G	1290
E.maclovinus	. . . . T	1290
N.coriiceps	C C T G A T G C C C A G C T G T T T G A G G G G C A G G A G	1320
C.aceratus	. . G	1320
E.maclovinus	. . . . G	1320

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N.coriiceps     G A G G A G G A G C A G C T G A A C A G A G A G A T G C C A 1350
C.aceratus      . . . . . . C . . . . . - - - . . . . . . . . . . . . . . 1347
E.maclovinus    . . . . . . C . . . T . - - - . . . . . G . . . . . . . . 1347

N.coriiceps     G A G G A C A T C G T G A G G C G C C A A C T T A T A A A C 1380
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1377
E.maclovinus    . . . . . . . . . . . . A . . . . . A . . . . . . . C . . . 1377

N.coriiceps     A A C C T G G C C A A G C A C G T C C T C T T C A C G G C T 1410
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1407
E.maclovinus    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1407

N.coriiceps     A A T A A G T C A T C A G C G A T C A T G T C C A C C C A C 1440
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1437
E.maclovinus    . . C . . . . . C . . . . . . . . . . . . . . . . . . . . 1437

N.coriiceps     A T T A T A G C C T G T C T C C T G C T G T A T A G A C A C 1470
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . 1467
E.maclovinus    . . C . . . . . . . . . . . . . . . . . . . . . C . . . . . 1467

N.coriiceps     A G A C A G G G G G T G G T G C T G T C C A A G T T G G T G 1500
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1497
E.maclovinus    . . . . . . . . . . . . . . G . . . . . . . . . . . . . . 1497

N.coriiceps     G A A G A C T T C T T C A A C A T G A A G G A G G A G A T C 1530
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1527
E.maclovinus    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 1527

N.coriiceps     C T G T C A C G G G A C T T T G A T C T G G G C T T C T C G 1560
C.aceratus      . . T . . . . . . . . . . . . . . . C . . . . . . . . . . 1557
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E.maclovinus	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	1557
N.coriiceps	G	G	G	A	A	C	T	C	T	G	A	G	G	A	T	G	T	T	G	T	G	G	T	G	C	G	T	G	C	C	1590
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1587
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	1587
N.coriiceps	C	T	C	C	A	C	C	T	G	C	T	G	G	G	A	A	A	C	T	G	C	G	T	C	A	A	C	G	T	G	1620
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1617
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	1617
N.coriiceps	A	C	C	A	G	C	A	G	C	G	C	T	A	A	C	C	G	C	A	A	C	G	G	A	G	A	G	T	T	C	1650
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1647
E.maclovinus	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	1647
N.coriiceps	A	C	C	A	T	C	G	C	G	C	C	G	A	G	C	C	A	G	A	C	T	G	T	G	C	C	A	G	C	G	1680
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1677
E.maclovinus	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	1677
N.coriiceps	C	T	T	T	T	C	G	A	G	C	T	C	A	A	C	T	T	C	T	A	C	A	G	C	A	A	C	G	G	C	1710
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1707
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	1707
N.coriiceps	C	T	T	T	T	C	C	A	T	G	T	C	T	T	C	A	T	T	T	C	T	G	A	T	G	C	A	A	T	C	1740
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1737
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	1737
N.coriiceps	A	T	C	G	C	C	T	G	C	A	G	C	A	T	C	C	T	G	T	C	C	C	T	G	C	A	G	C	G	A	1770
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1767
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	1767

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N.coriiceps     G A G C T G G T G G C T G A A T C A G A G T C C G A C C A C  1800
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . T . . . . C .  1797
E.maclovinus    . . A . . . . . . . . . . . . . . . . . . . . . T . . . . . .  1797

N.coriiceps     C C C C C C G A G T G T C T C A G C A G C C T C C C C C T C  1830
C.aceratus      . . . . . . . . . G . . . . . . . . . . . . . . . . . . . .  1827
E.maclovinus    . . . . . . . . . T G . . . . . . . . . . . . . . . T . . .  1827

N.coriiceps     A G T C A G G A G A G A C T C A T C C G A A A G G C C G C C  1860
C.aceratus      . . . . . . . . . . . G . . . . . . . . . . . . . . . . . .  1857
E.maclovinus    . . . . . . . . . . . . . . . . . . . . A . . . . . . . . .  1857

N.coriiceps     G G G C T C T C T C A C T T C C T T G T C A A T G A A G T G  1890
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  1887
E.maclovinus    . . . . . . . . . A . . . . . . . . . . . . . . . . . . . .  1887

N.coriiceps     A C A C T G G C A C C A C C A T G T C A G A C T A T T T A C  1920
C.aceratus      . . . . . . . . . . . . . . . C . . . . . . . . . . . . . .  1917
E.maclovinus    . . G . . . . . . . . . . . . C . . . . . . . . . . . . . .  1917

N.coriiceps     C A G G T T T T C C A T G A C G C A G T A G C G C G G C T C  1950
C.aceratus      . . . . . . . . . . . . . . . . . . . A . . . . A . . . . .  1947
E.maclovinus    . . . . . . . . . . . . . . . . . . . . . C A . C . . . . .  1947

N.coriiceps     A T T C A A T A T G G A G T T C T C T A C G T A G C A G A G  1980
C.aceratus      . . C . . . . . . . . . . . . . . . . . . . . . . . . . . .  1977
E.maclovinus    . . C . . G . . . . . . . . . . . . . . . . . . . . . . . .  1977

N.coriiceps     G A G G A C C A G G A G G A A C T G A G T C C C A G C C C C  2010
C.aceratus      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  2007
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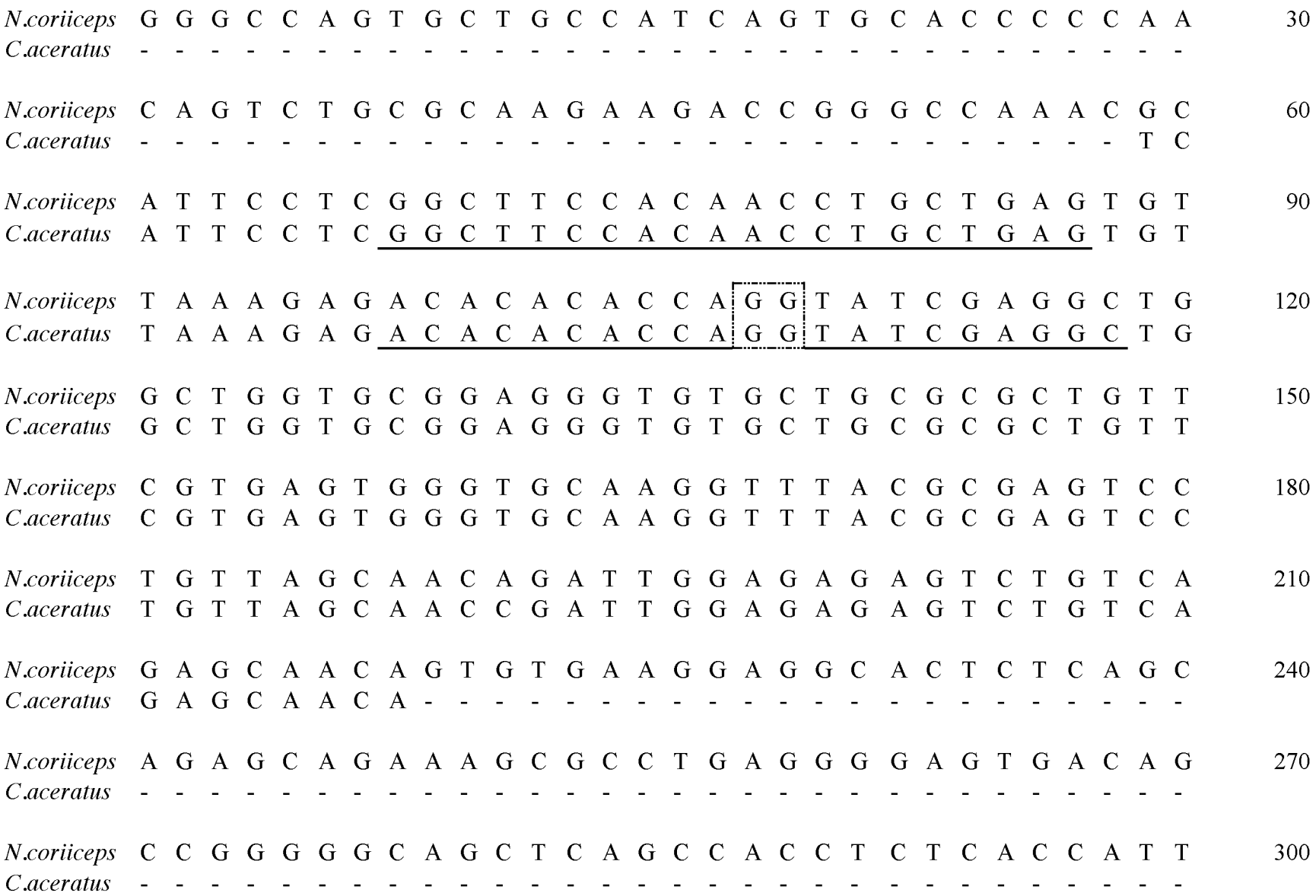
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2007
N.coriiceps	A	C	A	G	A	C	G	A	A	C	C	T	T	G	G	C	C	C	A	A	A	A	A	G	T	T	C	C	C	A				2040
C.aceratus	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2037
E.maclovinus	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2037
N.coriiceps	G	A	G	C	C	C	C	T	T	T	C	C	T	G	G	A	G	A	A	G	T	G	A	C	G	A	G	G	A	G				2070
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2067
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	2067
N.coriiceps	G	A	T	G	A	G	G	A	C	A	G	T	G	A	C	T	T	T	G	G	A	G	A	G	G	A	G	C	A	G				2100
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2097
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2097
N.coriiceps	A	G	A	G	A	T	C	G	C	T	A	T	C	T	A	A	A	G	G	T	G	A	G	C	G	T	T	T	C	C				2130
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2127
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2127
N.coriiceps	G	C	A	G	A	G	C	A	T	C	A	G	G	A	G	T	T	C	T	T	C	G	T	C	T	T	C	C	T	A				2160
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	2157
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2157
N.coriiceps	C	A	G	C	G	A	C	T	C	G	T	C	A	G	C	C	C	C	G	T	G	C	T	G	G	A	G	G	C	C				2190
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	2187
E.maclovinus	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2187
N.coriiceps	T	A	C	A	G	C	G	G	C	G	C	C	G	C	C	A	T	C	T	T	T	G	T	C	C	A	C	A	G	T				2220
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2217
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2217

N.coriiceps	C	T	G	A	G	T	C	A	G	C	C	C	A	T	G	G	C	C	G	A	G	T	C	C	G	A	C	T	A	C	2250
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2247
E.maclovinus	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2247
N.coriiceps	A	C	C	C	A	G	C	G	G	C	T	C	T	T	C	A	G	A	T	A	C	C	T	G	C	T	C	A	C	A	2280
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2277
E.maclovinus	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2277
N.coriiceps	C	G	C	A	C	A	G	A	G	A	G	A	G	G	A	G	T	G	G	C	T	G	C	T	T	A	T	G	G	T	2310
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2307
E.maclovinus	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2307
N.coriiceps	G	A	A	A	G	T	G	C	A	A	C	T	C	A	T	T	A	C	C	T	G	G	T	T	A	A	G	A	A	C	2340
C.aceratus	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2337
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2337
N.coriiceps	A	C	A	G	T	G	A	G	G	A	C	A	T	T	C	A	A	A	G	A	G	C	T	T	G	G	G	G	T	C	2370
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	2367
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2367
N.coriiceps	C	T	T	A	A	G	C	A	G	A	G	A	A	A	A	G	A	G	A	A	C	C	A	G	G	T	G	A	C	G	2400
C.aceratus	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2397
E.maclovinus	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2397
N.coriiceps	A	C	T	C	T	G	G	A	G	C	T	G	A	G	C	A	G	C	A	C	C	T	T	T	C	T	A	C	C	T	2430
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2427
E.maclovinus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2427
N.coriiceps	C	A	G	G	C	C	A	A	T	C	G	G	A	A	C	A	A	A	C	T	A	C	T	G	C	A	G	T	A	C	2460
C.aceratus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2457

E.maclovinus	. . . . .	2457
N.coriiceps	A T C T T G G G T T T C A C C C T G C T G T G A	2484
C.aceratus	. . . . . G	2481
E.maclovinus	. . . . .	2481

Figure 1.2. (pages 25-36) Predicted cDNA sequence of GPAT1 in three notothenioids species. Annealing sites for primers used in qRT-PCR are underlined in *N. coriiceps* and *C. aceratus*.


N.coriiceps G G G C C A G T G C T G C C A T C A G T G C A C C C C C A A 30
C.aceratus -
N.coriiceps C A G T C T G C G C A A G A A G A C C G G G C C A A A C G C 60
C.aceratus - T C
N.coriiceps A T T C C T C G G C T T C C A C A A C C T G C T G A G T G T 90
C.aceratus A T T C C T C G G C T T C C A C A A C C T G C T G A G T G T
N.coriiceps T A A A G A G A C A C A C A C C A G G T A T C G A G G C T G 120
C.aceratus T A A A G A G A C A C A C A C C A G G T A T C G A G G C T G
N.coriiceps G C T G G T G C G G A G G G T G T G C T G C G C G C T G T T 150
C.aceratus G C T G G T G C G G A G G G T G T G C T G C G C G C T G T T
N.coriiceps C G T G A G T G G G T G C A A G G T T T A C G C G A G T C C 180
C.aceratus C G T G A G T G G G T G C A A G G T T T A C G C G A G T C C
N.coriiceps T G T T A G C A A C A G A T T G G A G A G A G T C T G T C A 210
C.aceratus T G T T A G C A A C C G A T T G G A G A G A G T C T G T C A
N.coriiceps G A G C A A C A G T G T G A A G G A G G C A C T C T C A G C 240
C.aceratus G A G C A A C A -
N.coriiceps A G A G C A G A A A G C G C C T G A G G G G A G T G A C A G 270
C.aceratus -
N.coriiceps C C G G G G G C A G C T C A G C C A C C T C T C A C C A T T 300
C.aceratus -

N.coriiceps C C T C C C C C T C A T T A A C A C C A G C A T C T C C C C 330
C.aceratus -

N.coriiceps T G G C C T C T T A C G A T T C C T G A G C T G G G T G C T 360
C.aceratus - - - - - - - - - - - - G A T T C C T G A A C T G G A T G C T

N.coriiceps G C T G A A G A T G T T T T C T T C C A T G T T T G G C A G 390
C.aceratus G C T G A A G A T G T T T G C T T C C G W G T T T G G C A R

N.coriiceps T A T T C A A G C T A A C C T C A A C C A T C T G C C G G C 420
C.aceratus T A T T C A A G T T A A C C T C A A C C A T C T G C C G G C

N.coriiceps T T T G C A C A G A G C T T C A C A A G A G G G A T C G G T 450
C.aceratus T T T G C A C A N A G C T T C A C A A G A G G R A A C G G T

N.coriiceps G C T G G T T T A T G T G T A T T C A C A T C A G A G C G T 480
C.aceratus G C T G G T T T A T G T G T A T T C A C A T C A G A G C G T

N.coriiceps C G T G G A C T G T G C T C T C A T C C C T C T G G T G C T 510
C.aceratus C N T G G A C T G A G C T C T C A T C C C T C T G G T G C T

N.coriiceps C T T C T G T C A C A G C C T C A G A G T G C C A T A C A C 540
C.aceratus C T T C T G T C A C A G C C T C A G A G T G C C A T A C A C

N.coriiceps T G T T T T C C C G C T C C A C A T T A C C A G C T C C T T 570
C.aceratus T G T T T T C C C G C T C C A C A T T A - - - - - - - - - - -

N.coriiceps C A T A A G A T C A A T C C T G C A G A A A G T C G G T G T 600
C.aceratus -

N.coriiceps C C T C G T A C C A C C T G C A C T G A C G A A G G A G G A 630
C.aceratus -

N.coriiceps T G C T G A G A C T G A C A G A C T G C A C G C C C C G T 660
C.aceratus -

N.coriiceps C A T G A C C T C T C T G G T A C G T G A G C T G C T A C A 690
C.aceratus - - - - - - - - - - - - T G G T A C G T G A G C T G C T A C A

N.coriiceps G G A G G G T C A G G C C C T A A G T G T C G G C G T G G C 720
C.aceratus G G A G G G T C A G G C C C T A A G T G T C G G C G T G G C

N.coriiceps A G C G G A G T C T G G - C C G G G G C G G C C A G T G G C 750
C.aceratus A C C G G A G T C T G G C C C G G G G C G G C C A G T G G C

N.coriiceps T G G C T T C C A T C A G A C A A G T C A T C A A A G A A G 780
C.aceratus T G G C T T C C A T C A G A C A A G T C A T C A A A G A A G

N.coriiceps G A T C T G T C C C C G A T G T C A G T C T G G T A C C T G 810
C.aceratus G A T C T G T C C C C G A T G T C A G T C T G G T A C C T G

N.coriiceps T G G G C A T C T C C T A C G A C T G T G T G C C A A A G A 840
C.aceratus T G G G C A T C T C C T A C G A C T G T G T G C C A A A G A

N.coriiceps G C A A C A A A C A G A T T G G C C T C A G C T C T G T G T 870
C.aceratus G C A A C A C A C A G G T T G G C C T C A G C T C T G T G T

N.coriiceps T G C G C T G G C T G T G C T C T C T C C T C T G G A G G A 900
C.aceratus T G C A C T G G C T G T G C T C T C T C C T C T G G A G G A

N.coriiceps G A C C G G A A G G A A T T G T G A G G A T C G A C C T C G 930
C.aceratus G A C C G G A A G G A A T T G T G A G G A T C G A C C T C G

N.coriiceps C C C A G C C T T T T T C T C T C A A G G A G A T G T G C G 960
C.aceratus T C C A G C C T T T T T C T C T C A A G G A G A T G T G C G

N.coriiceps A G T C A G G G A G G T G C A G A G T A G A T C A A T G G C 990
C.aceratus A G T C A G G G A G G T G C A G A G T A G A T G A A T G G C

N.coriiceps T C C C T C T A C A G G A C C T G T T G C T G C C A G T T A 1020
C.aceratus T C C C T C T A C A G G A C C T G T T G C T G C C G G T T A

N.coriiceps T C C T C A A C A A C A G A A C T G A C A G T G T G C T T G 1050
C.aceratus T C C T C A A C A A C A G A C C T G A C A G T G T G T T T G

N.coriiceps G G C G G A G G A G G A T G T C G T G G C T T C T T C C T T 1080
C.aceratus G G C G G A G G A G G A T G T C G T G G A T T C T T C C T T

N.coriiceps C T C C T C A T T A T G C G T C T G A A C T C A A A G A G C 1110
C.aceratus C T C C T C A T T A T G C G T C T G A A C T C A A A G A G C

N.coriiceps C A G C T G A T C C G G A G A G A G A C C T G A G C A C C G 1140
C.aceratus C A G C T G A T C C G G A G A G A G A C C T G A G C A C C G

N.coriiceps C C G T C A T C C T C C A C C T C C T C T T C T C G G C A G 1170
C.aceratus C C G T C A T C C T C C A C C T C C T C T T C T C T G C A G

N.coriiceps C C T C C T G C T C G G C T G T G A T G T C C A C C G G T C 1200
C.aceratus C C T C C T G C T C G G C T G T G A T G T C C A C C G G T C

N.coriiceps T G G T G T C C A G T C T T T T G C T G T A C A G A C A C C 1230
C.aceratus T G G T G T C C A G T C T T T T G C T G T A C A G A C A C C

N.coriiceps A C A A G G G T G T G A G T G C C T C T G C C C T G T G T C 1260
C.aceratus A C A A G G G T G T G A G T G C C T C T G C C C T G T G T C

N.coriiceps A G G A T G T G G C C T G G C T C A C A G A A G A G C T C T 1290
C.aceratus G G G A T G T -

N.coriiceps T G T T C A G A A A C A A G G A C G T G G G C T T C G G C G 1320
C.aceratus -

N.coriiceps G 1321
C.aceratus -

Figure 1.3. (pages 37-41) GPAT2 partial cDNA sequence obtained in Antarctic notothenioids. GPAT2 cDNA was sequenced from heart ventricles of Antarctic notothenioids *Chaenocephalus aceratus* and *Notothenia coriiceps*. Gene specific primers used to measure GPAT2 mRNA levels are underlined. The reverse primer was designed over a splice site indicated by dashed lines. Numbering is to show the length of sequence obtained, not the nucleotide position number.

N. coriiceps	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	A	L	S	D	G	6
C. aceratus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	A	L	S	D	G	6
E. macolvinus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	E	L	S	D	G	6
G.aculeatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	E	R	K	G	T	A	E	N	M	D	L	S	D	G	6
T.rubripes	M	N	H	L	S	A	A	L	K	L	V	L	Y	D	L	G	G	G	E	R	T	T	E	N	M	D	M	S	D	G	6
P.nyererei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	E	L	S	D	G	6
O.niloticus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	E	L	S	D	G	6
M.zebra	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	E	L	S	D	G	6
G.gallus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	D	E	T	A	L	S	L	G	T	I	D	V	13
M.gallopavo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	D	E	T	A	L	S	L	G	T	I	D	V	13
H.sapiens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	D	E	S	A	L	T	L	G	T	I	D	V	13
M.musculus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	E	E	S	S	V	T	V	G	T	I	D	V	13
R.norvegicus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	E	E	S	S	V	T	I	G	T	I	D	V	13
B.taurus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	M	D	E	S	A	L	T	L	G	T	I	D	V	13

N. coriiceps	L	L	L	Q	V	N	N	G	E	Q	W	T	N	R	W	K	H	P	N	D	D	S	-	D	R	S	T	S	P	S	35
C. aceratus	L	L	L	Q	V	N	N	G	E	Q	W	T	N	R	W	K	H	P	N	D	D	S	-	D	R	S	T	S	P	S	35
E. macolvinus	L	L	L	Q	V	N	N	G	E	Q	W	T	N	R	W	K	H	P	N	D	D	S	-	D	R	S	T	S	P	S	35
G.aculeatus	L	L	L	Q	V	N	N	G	E	Q	W	C	N	R	W	K	H	P	N	D	D	S	-	D	R	S	T	S	P	S	35
T.rubripes	L	L	L	Q	V	N	N	G	E	Q	W	C	N	R	W	K	H	P	N	D	D	L	-	D	R	S	T	S	P	S	35
P.nyererei	L	L	L	Q	V	S	N	G	E	Q	W	C	N	R	W	K	H	P	N	E	D	S	-	D	R	S	T	S	P	S	35
O.niloticus	L	L	L	Q	V	N	N	G	E	Q	W	C	N	R	W	K	H	P	N	E	E	S	-	D	R	S	T	S	P	S	35
M.zebra	L	L	L	Q	V	S	N	G	E	Q	W	C	N	R	W	K	H	P	N	E	D	S	-	D	R	S	T	S	P	S	35
G.gallus	S	Y	L	S	T	S	A	-	E	C	S	V	S	R	C	K	H	S	N	E	E	W	G	E	C	N	S	R	P	T	42
M.gallopavo	S	Y	L	S	T	S	A	-	E	C	S	V	S	R	C	K	H	S	N	E	E	W	G	E	C	N	S	R	P	T	42
H.sapiens	S	Y	L	P	H	S	S	-	E	Y	S	V	G	R	C	K	H	T	S	E	E	W	G	E	C	G	F	R	P	T	42
M.musculus	S	Y	L	P	S	S	S	-	E	Y	S	L	G	R	C	K	H	T	S	E	D	W	V	D	C	G	F	K	P	T	42
R.norvegicus	S	Y	L	P	N	S	S	-	E	Y	S	L	G	R	C	K	H	T	N	E	D	W	V	D	C	G	F	K	P	T	42
B.taurus	S	Y	L	P	N	S	S	-	E	Y	S	I	G	R	C	K	H	A	T	E	E	W	G	E	C	G	S	R	P	T	42

N. coriiceps	V	L	R	C	V	A	S	T	W	K	E	G	L
C. aceratus	V	L	R	C	V	A	S	T	W	K	E	G	L
E. macolvinus	V	L	R	C	V	A	S	T	W	K	E	G	L
G.aculeatus	V	L	R	C	V	A	S	T	W	K	E	G	L
T.rubripes	V	L	R	C	V	A	S	T	W	K	E	G	L
P.nyererei	V	L	R	N	V	A	S	T	W	K	E	G	L
O.niloticus	V	L	R	N	V	A	S	T	W	K	E	G	L
M.zebra	V	L	R	N	V	A	S	T	W	K	E	G	L
G.gallus	L	F	R	S	A	T	L	K	W	K	E	T	L
M.gallopavo	L	F	R	S	A	T	L	K	W	K	E	T	L
H.sapiens	I	F	R	S	A	T	L	K	W	K	E	S	L
M.musculus	F	F	R	S	A	T	L	K	W	K	E	S	L
R.norvegicus	F	F	R	S	A	T	L	K	W	K	E	S	L
B.taurus	V	F	R	S	A	T	L	K	W	K	E	S	L

N. coriiceps	Q	S	W	E	K	L	F	N	P	S	I	P	S
C. aceratus	Q	S	W	E	K	L	F	N	P	S	I	P	S
E. macolvinus	Q	S	W	E	K	L	F	N	P	S	I	P	S
G.aculeatus	Q	S	W	E	K	L	F	N	P	S	I	P	S
T.rubripes	Q	S	W	E	K	L	F	N	P	S	I	P	S
P.nyererei	Q	S	W	E	R	L	F	N	P	S	I	P	S
O.niloticus	Q	S	W	E	R	L	F	N	P	S	I	P	S
M.zebra	Q	S	W	E	R	L	F	N	P	S	I	P	S
G.gallus	Q	S	R	D	N	F	F	N	A	S	I	P	S
M.gallopavo	Q	S	R	D	N	F	F	N	A	S	I	P	S
H.sapiens	Q	S	W	D	K	F	F	N	P	S	I	P	S
M.musculus	Q	S	W	E	R	F	F	N	P	S	I	P	S
R.norvegicus	Q	S	W	E	R	F	F	N	P	S	I	P	S
B.taurus	Q	S	W	D	K	F	F	N	P	S	I	P	S

L	S	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
L	S	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
L	S	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
L	N	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
L	N	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
L	N	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
L	N	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
L	N	R	K	R	P	F	V	G	R	C	C	H	S	C	T	P	65
S	-	R	K	R	P	F	V	G	R	C	C	Y	V	C	T	P	71
S	-	R	K	R	P	F	V	G	R	C	C	Y	V	C	T	P	71
M	S	R	K	R	P	F	V	G	R	C	C	Y	S	C	T	P	72
M	S	R	K	R	P	F	V	G	R	C	C	Y	S	C	T	P	72
M	S	R	K	R	P	F	V	G	R	C	C	Y	S	C	T	P	72
M	S	R	K	R	P	F	V	G	R	C	C	Y	S	C	T	P	72

L	G	L	R	N	V	I	F	I	N	E	T	H	T	-	-	R	93
L	G	L	R	N	V	I	F	I	N	E	T	H	T	-	-	R	93
L	G	L	R	N	V	I	F	I	N	E	T	H	T	-	-	R	93
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	R	C	R	95
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	93
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	93
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	93
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	93
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	99
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	99
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	100
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	100
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	100
L	G	L	R	N	V	I	Y	I	N	E	T	H	T	-	-	R	100

N. coriiceps	Q	R	G	W	L	A	R	R	L	S	Y	V	L
C. aceratus	Q	R	G	W	L	A	R	R	L	S	Y	V	L
E. macolvinus	Q	R	G	W	L	A	R	R	L	S	Y	V	L
G.aculeatus	Q	R	G	W	L	A	R	R	L	S	Y	V	L
T.rubripes	Q	R	G	W	L	A	R	R	L	S	Y	V	L
P.nyererei	Q	R	G	W	L	A	R	R	L	S	Y	V	L
O.niloticus	Q	R	G	W	L	A	R	R	L	S	Y	V	L
M.zebra	Q	R	G	W	L	A	R	R	L	S	Y	V	L
G.gallus	Y	R	G	W	L	A	R	R	L	C	Y	V	L
M.gallopavo	Y	R	G	W	L	A	R	R	L	C	Y	V	L
H.sapiens	H	R	G	W	L	A	R	R	L	S	Y	V	L
M.musculus	H	R	G	W	L	A	R	R	L	S	Y	I	L
R.norvegicus	H	R	G	W	L	A	R	R	L	S	Y	I	L
B.taurus	H	R	G	W	L	A	R	R	L	S	Y	V	L

N. coriiceps	D	N	V	L	N	N	S	R	V	E	S	A	I
C. aceratus	D	N	V	L	N	N	S	R	V	E	S	A	I
E. macolvinus	D	N	V	L	N	N	C	R	V	E	S	A	I
G.aculeatus	D	N	V	L	N	S	N	G	V	E	C	S	I
T.rubripes	D	N	V	L	N	N	K	K	V	E	N	A	I
P.nyererei	D	N	V	L	N	N	S	R	V	E	D	A	I
O.niloticus	D	N	V	L	N	N	S	R	V	E	N	A	I
M.zebra	D	N	V	L	N	N	S	R	V	E	D	A	I
G.gallus	E	N	V	L	N	S	S	R	V	Q	K	A	I
M.gallopavo	E	N	V	L	N	S	S	R	V	Q	K	A	I
H.sapiens	E	N	V	L	N	S	S	R	V	Q	E	A	I
M.musculus	E	N	V	L	S	S	S	R	V	Q	E	A	I
R.norvegicus	D	N	V	L	N	S	S	R	V	Q	E	A	I
B.taurus	E	N	V	L	N	S	S	R	V	Q	E	A	I

F	V	M	E	R	D	V	N	K	D	M	F	T	R	N	V	V	123
F	V	M	E	R	D	V	N	K	D	M	F	T	R	N	V	V	123
F	V	M	E	R	D	V	N	K	D	M	F	T	R	N	V	V	123
F	V	M	E	R	D	V	N	K	D	M	F	T	R	N	V	V	125
F	V	M	E	R	D	V	N	K	D	M	F	T	R	N	I	V	123
F	V	V	E	R	D	V	H	K	D	M	F	T	R	N	M	V	123
F	V	V	E	R	D	V	H	K	D	M	F	T	R	N	V	V	123
F	V	V	E	R	D	V	H	K	D	M	F	T	R	N	V	V	123
F	V	L	E	R	D	V	H	K	G	M	F	A	K	N	L	T	129
F	V	L	E	R	D	A	H	K	G	M	F	A	K	N	L	T	129
F	I	Q	E	R	D	V	H	K	G	M	F	A	T	N	V	T	130
F	V	Q	E	R	D	V	H	K	G	M	F	A	T	S	V	T	130
F	V	Q	E	R	D	V	H	K	G	M	F	A	T	S	I	T	130
F	I	Q	E	R	D	V	H	K	G	M	F	A	T	N	V	T	130

A	N	V	A	A	D	V	D	A	A	G	T	Q	P	G	Q	E	153
A	D	V	A	T	D	L	D	A	A	G	T	Q	P	G	Q	E	153
A	N	V	A	T	D	L	D	A	A	G	T	Q	P	G	Q	E	153
A	E	V	A	S	D	L	D	A	A	G	R	H	P	G	Q	E	155
E	K	V	A	A	D	V	D	A	A	S	S	Q	P	G	K	K	153
V	T	V	A	T	D	L	D	A	A	A	S	Q	P	G	Q	E	153
V	T	V	A	T	D	L	D	A	A	A	S	Q	P	G	Q	E	153
V	T	V	A	T	D	L	D	A	A	A	S	Q	P	G	Q	E	153
V	D	E	A	S	E	P	S	V	P	G	S	F	A	Q	M	D	159
V	D	E	A	S	E	P	S	V	P	G	S	F	A	Q	V	D	159
A	E	V	A	A	E	L	N	P	D	G	S	-	A	Q	Q	Q	159
A	E	V	A	A	E	L	N	P	D	G	S	-	A	Q	Q	Q	159
A	E	V	A	A	E	L	N	P	D	G	S	-	A	Q	Q	Q	159
A	E	V	A	G	E	L	N	P	D	G	S	-	A	Q	Q	Q	159

N. coriiceps	H	K	A	V	R	K	V	K	Q	K	A	R	A	F	L
C. aceratus	H	K	A	V	R	K	V	K	Q	K	A	R	A	F	L
E. macolvinus	H	K	A	V	R	K	V	K	Q	K	A	R	S	F	L
G.aculeatus	H	K	A	I	S	K	V	K	Q	K	A	R	A	F	L
T.rubripes	Q	K	G	V	S	K	V	K	Q	K	A	R	A	C	L
P.nyererei	H	K	A	V	S	K	V	K	Q	K	A	R	A	F	L
O.niloticus	H	K	A	V	S	K	V	K	Q	K	A	R	A	F	L
M.zebra	H	K	A	V	S	K	V	K	Q	K	A	R	A	F	L
G.gallus	P	K	A	I	N	K	V	K	K	K	A	R	K	I	L
M.gallopavo	P	K	A	I	N	K	V	K	K	K	A	R	K	I	L
H.sapiens	S	K	A	V	N	K	V	K	K	K	A	K	R	I	L
M.musculus	S	K	A	I	Q	K	V	K	R	K	A	R	K	I	L
R.norvegicus	S	K	A	I	Q	K	V	K	R	K	A	R	K	I	L
B.taurus	S	K	A	V	N	K	V	K	K	K	A	R	K	I	L

N. coriiceps	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
C. aceratus	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
E. macolvinus	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
G.aculeatus	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
T.rubripes	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
P.nyererei	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
O.niloticus	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
M.zebra	G	W	V	L	L	R	L	F	N	G	F	F	W	S	I
G.gallus	G	W	V	L	L	K	L	F	N	S	F	F	W	N	I
M.gallopavo	G	W	V	L	L	K	L	F	N	S	F	F	W	N	I
H.sapiens	G	W	V	L	L	K	L	F	N	S	F	F	W	N	I
M.musculus	G	W	V	L	L	K	L	F	N	S	F	F	W	N	I
R.norvegicus	G	W	V	L	L	K	L	F	N	S	F	F	W	N	I
B.taurus	G	W	V	L	L	K	L	F	N	S	F	F	W	N	I

Q	E	M	V	A	N	I	S	P	A	F	I	R	L	T	183
Q	E	M	V	A	N	I	S	P	A	F	I	R	L	T	183
Q	E	M	V	A	N	I	S	P	A	F	I	R	L	T	183
Q	E	M	V	A	N	I	S	P	A	F	I	R	L	T	185
Q	E	M	V	A	N	V	S	P	A	F	I	R	L	T	183
Q	E	M	V	A	N	I	S	P	A	F	I	R	L	T	183
Q	E	M	V	A	N	I	S	P	A	F	I	R	L	T	183
Q	E	M	V	A	N	I	S	P	A	F	I	R	L	T	183
Q	E	M	V	A	N	V	S	P	A	L	I	R	L	T	189
Q	E	M	V	A	N	V	S	P	A	L	I	R	L	T	189
Q	E	M	V	A	T	V	S	P	A	M	I	R	L	T	189
Q	E	M	V	A	T	V	S	P	G	M	I	R	L	T	189
Q	E	M	V	A	T	V	S	P	G	M	I	R	L	T	189
Q	E	M	V	A	T	V	S	P	A	M	I	R	L	T	189

Q	I	H	K	G	Q	L	E	I	V	K	K	A	A	T	213
Q	I	H	K	G	Q	L	E	M	V	K	K	A	A	T	213
Q	I	H	K	G	Q	L	E	M	V	K	K	A	A	T	213
Q	I	H	K	G	Q	L	E	M	V	K	K	A	A	T	215
Q	I	H	K	G	Q	L	E	M	V	K	K	A	A	T	213
Q	I	H	K	G	Q	L	E	M	V	K	K	A	A	T	213
Q	I	H	K	G	Q	L	E	M	V	K	K	A	A	T	213
Q	I	H	K	G	Q	L	E	M	V	K	K	A	A	T	213
Q	I	H	R	G	Q	I	E	M	V	K	-	A	A	T	218
Q	V	H	R	G	Q	I	E	M	V	K	-	A	A	T	218
Q	I	H	K	G	Q	L	E	M	V	K	-	A	A	T	218
Q	I	H	K	G	Q	L	E	M	V	K	-	A	A	T	218
Q	I	H	K	G	Q	L	E	M	V	K	-	A	A	T	218
Q	I	H	K	G	Q	L	E	M	V	K	-	A	A	T	218

N. coriiceps	E	Q	N	V	P	M	V	F	L	P	V	H	K	S
C. aceratus	E	Q	N	V	P	M	V	F	L	P	V	H	K	S
E. macolvinus	E	Q	N	V	P	M	V	F	L	P	V	H	K	S
G.aculeatus	E	Q	N	V	P	M	V	F	L	P	V	H	K	S
T.rubripes	E	Q	N	V	P	M	I	F	L	P	V	H	K	S
P.nyererei	E	Q	N	V	P	M	V	F	L	P	V	H	K	S
O.niloticus	E	Q	N	V	P	M	V	F	L	P	V	H	K	S
M.zebra	E	Q	N	V	P	M	V	F	L	P	V	H	K	S
G.gallus	E	M	N	L	P	L	I	F	L	P	V	H	K	S
M.gallopavo	E	M	N	L	P	L	I	F	L	P	V	H	K	S
H.sapiens	E	T	N	L	P	L	L	F	L	P	V	H	R	S
M.musculus	E	T	N	L	P	L	L	F	L	P	V	H	R	S
R.norvegicus	E	T	N	L	P	L	L	F	L	P	V	H	R	S
B.taurus	E	T	N	L	P	L	I	F	L	P	V	H	R	S

N. coriiceps	K	A	P	H	I	A	A	G	N	N	L	S	I	P
C. aceratus	K	A	P	H	I	A	A	G	N	N	L	S	I	P
E. macolvinus	K	A	P	H	I	A	A	G	N	N	L	S	I	P
G.aculeatus	K	A	P	H	I	A	A	G	N	N	L	S	I	P
T.rubripes	K	A	P	H	I	A	A	G	N	N	L	S	I	P
P.nyererei	K	A	P	H	I	A	A	G	N	N	L	S	I	P
O.niloticus	K	A	P	H	I	A	A	G	N	N	L	S	I	P
M.zebra	K	A	P	H	I	A	A	G	N	N	L	S	I	P
G.gallus	K	A	P	Y	I	A	A	G	N	N	L	N	I	P
M.gallopavo	K	A	P	Y	I	A	A	G	N	N	L	N	I	P
H.sapiens	K	A	P	Y	I	A	S	G	N	N	L	N	I	P
M.musculus	K	A	P	Y	I	A	S	G	N	N	L	N	I	P
R.norvegicus	K	A	P	Y	I	A	S	G	N	N	L	N	I	P
B.taurus	K	A	P	Y	I	A	S	G	N	N	L	N	I	P

H	I	D	Y	L	L	I	T	L	I	L	F	C	H	N	I	243
H	I	D	Y	L	L	I	T	L	I	L	F	C	H	N	I	243
H	I	D	Y	L	L	I	T	L	I	L	F	C	H	N	I	243
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H	I	D	Y	L	L	L	T	F	I	L	F	C	H	N	I	248
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V	F	S	T	L	I	H	K	L	G	G	F	F	I	R	R	278
I	F	S	T	L	I	H	K	L	G	G	F	F	I	R	R	278
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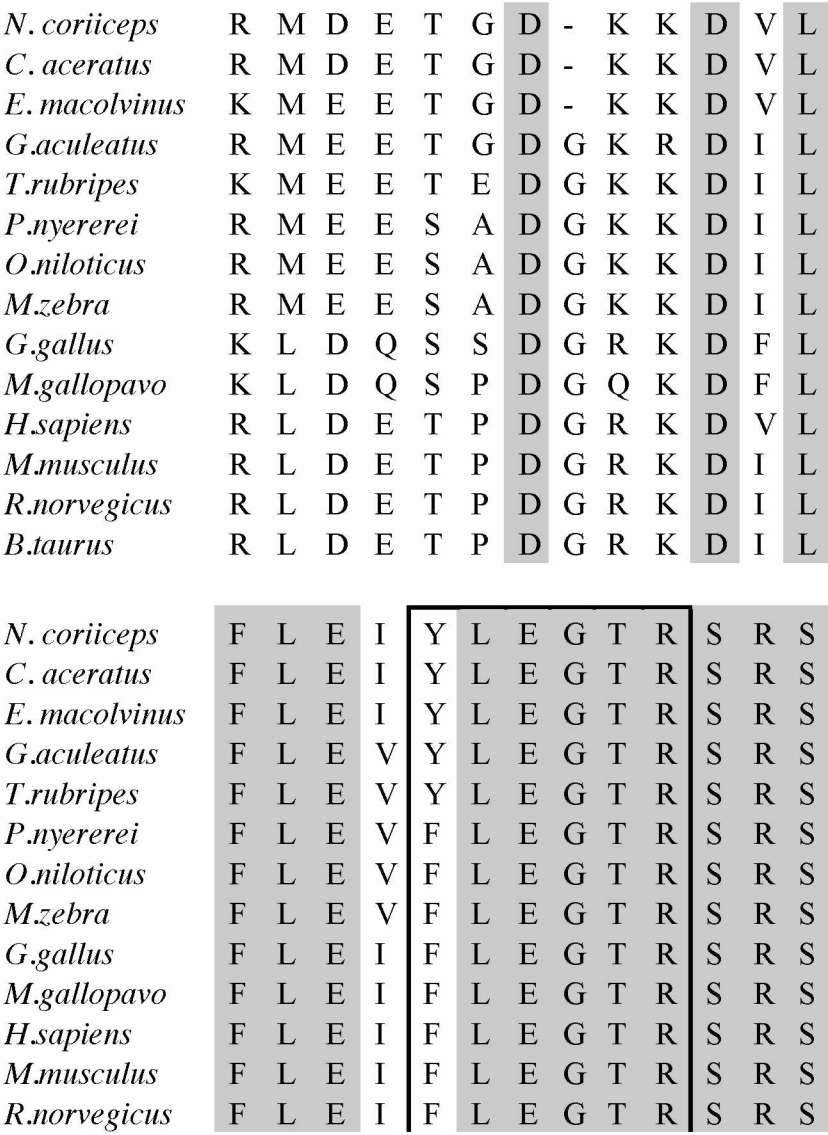
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G.aculeatus R M E E T G D G K R D I L
T.rubripes K M E E T E D G K K D I L
P.nyererei R M E E S A D G K K D I L
O.niloticus R M E E S A D G K K D I L
M.zebra R M E E S A D G K K D I L
G.gallus K L D Q S S D G R K D F L
M.gallopavo K L D Q S P D G Q K D F L
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G.aculeatus F L E V Y L E G T R S R S
T.rubripes F L E V Y L E G T R S R S
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H.sapiens F L E I F L E G T R S R S
M.musculus F L E I F L E G T R S R S
R.norvegicus F L E I F L E G T R S R S

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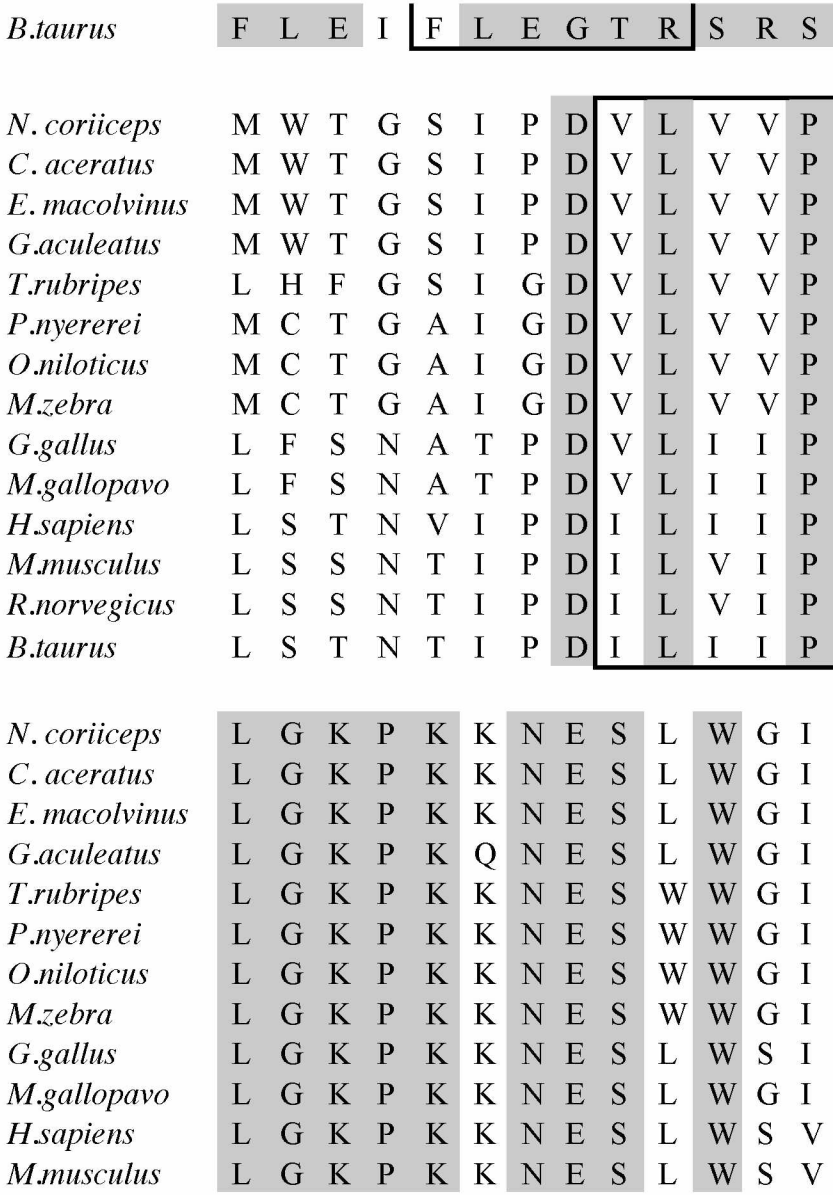
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E. macolvinus M W T G S I P D V L V V P
G.aculeatus M W T G S I P D V L V V P
T.rubripes L H F G S I G D V L V V P
P.nyererei M C T G A I G D V L V V P
O.niloticus M C T G A I G D V L V V P
M.zebra M C T G A I G D V L V V P
G.gallus L F S N A T P D V L I I P
M.gallopavo L F S N A T P D V L I I P
H.sapiens L S T N V I P D I L I I P
M.musculus L S S N T I P D I L V I P
R.norvegicus L S S N T I P D I L V I P
B.taurus L S T N T I P D I L I I P
N. coriiceps L G K P K K N E S L W G I
C. aceratus L G K P K K N E S L W G I
E. macolvinus L G K P K K N E S L W G I
G.aculeatus L G K P K Q N E S L W G I
T.rubripes L G K P K K N E S W W G I
P.nyererei L G K P K K N E S W W G I
O.niloticus L G K P K K N E S W W G I
M.zebra L G K P K K N E S W W G I
G.gallus L G K P K K N E S L W S I
M.gallopavo L G K P K K N E S L W G I
H.sapiens L G K P K K N E S L W S V
M.musculus L G K P K K N E S L W S V

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V	G	I	S	Y	D	R	I	L	E	G	N	Y	N	S	E	Q	362
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A	C	G	V	F	R	M	L	R	K	N	Y	G	C	V	R	V	392
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A	R	G	V	I	R	M	L	R	K	N	Y	G	Y	V	R	V	398

R.norvegicus	L	G	K	P	K	K	N	E	S	L	W	S	V	A	R
B.taurus	L	G	K	P	K	K	N	E	S	L	W	S	I	A	R

N. coriiceps	D	F	N	Q	P	F	S	L	K	E	Y	L	D	S	Q
C. aceratus	D	F	N	Q	P	F	S	L	K	E	Y	L	D	S	Q
E. macolvinus	D	F	N	Q	P	F	S	L	K	E	Y	L	D	S	Q
G.aculeatus	D	F	N	Q	P	F	S	L	K	E	Y	L	D	S	Q
T.rubripes	D	F	N	Q	P	F	S	L	K	E	Y	L	D	T	Q
P.nyererei	D	F	N	Q	P	F	S	L	K	E	Y	L	D	T	Q
O.niloticus	D	F	N	Q	P	F	S	L	K	E	Y	L	D	T	Q
M.zebra	D	F	N	Q	P	F	S	L	K	E	Y	L	D	T	Q
G.gallus	D	F	A	Q	P	F	S	L	K	E	Y	V	N	S	Q
M.gallopavo	D	F	A	Q	P	F	S	L	K	E	Y	V	N	S	Q
H.sapiens	D	F	A	Q	P	F	S	L	K	E	Y	L	E	S	Q
M.musculus	D	F	A	Q	P	F	S	L	K	E	Y	L	E	G	Q
R.norvegicus	D	F	A	Q	P	F	S	L	K	E	Y	L	E	G	Q
B.taurus	D	F	A	Q	P	F	S	L	K	E	Y	L	E	S	Q

N. coriiceps	M	P	I	I	I	S	A	Q	P	D	A	Q	L	F	E
C. aceratus	M	P	I	I	I	S	A	Q	P	D	A	Q	L	F	E
E. macolvinus	M	P	I	I	I	S	A	Q	P	D	A	Q	L	F	E
G.aculeatus	M	P	I	I	I	S	A	Q	P	D	A	Q	L	F	E
T.rubripes	L	P	T	I	I	S	A	Q	P	D	A	Q	L	F	E
P.nyererei	M	P	T	I	I	S	A	E	P	D	A	Q	L	F	D
O.niloticus	M	P	T	I	I	S	A	E	P	D	A	Q	L	F	D
M.zebra	M	P	T	I	I	S	A	E	P	D	A	Q	L	F	D
G.gallus	L	P	A	I	L	P	S	R	P	N	D	T	V	D	E
M.gallopavo	L	P	A	I	L	P	S	R	P	N	D	T	V	D	E
H.sapiens	L	P	A	I	L	P	S	R	P	S	D	A	A	D	E
M.musculus	L	P	A	I	L	P	S	R	P	N	D	V	A	D	E

G	V	I	R	M	L	R	K	N	Y	G	Y	V	R	V	398
G	V	I	R	M	L	R	K	N	Y	G	C	V	K	T	398

R	N	R	H	I	P	P	A	V	S	L	E	H	T	L	422
R	N	R	H	I	P	P	A	V	S	L	E	Q	T	L	422
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G	Q	E	E	E	E	Q	L	N	R	E	M	P	E	D	452
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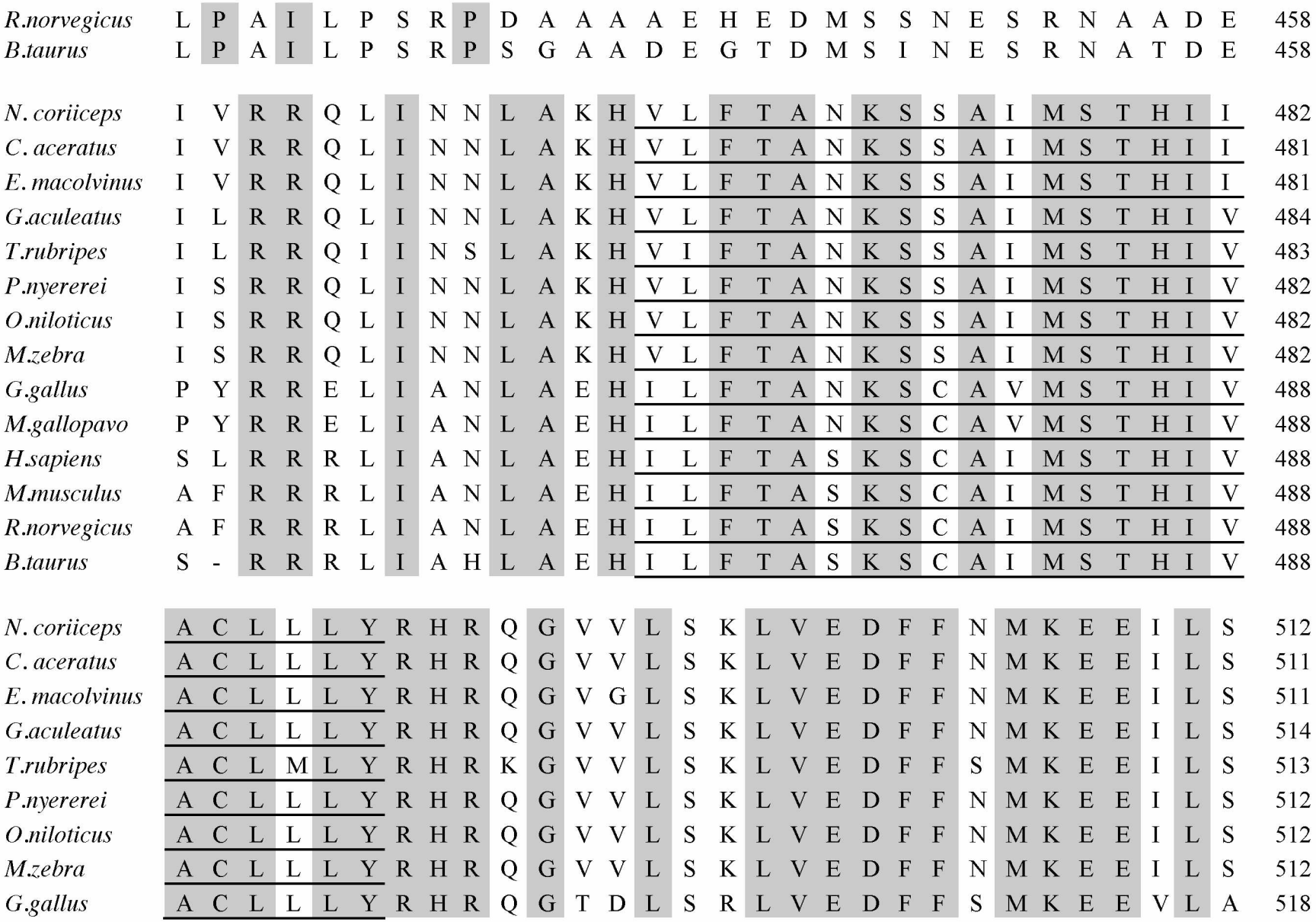
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N. coriiceps I V R R Q L I N N L A K H V L F T A N K S S A I M S T H I I 482
C. aceratus I V R R Q L I N N L A K H V L F T A N K S S A I M S T H I I 481
E. macolvinus I V R R Q L I N N L A K H V L F T A N K S S A I M S T H I I 481
G.aculeatus I L R R Q L I N N L A K H V L F T A N K S S A I M S T H I V 484
T.rubripes I L R R Q I I N S L A K H V I F T A N K S S A I M S T H I V 483
P.nyererei I S R R Q L I N N L A K H V L F T A N K S S A I M S T H I V 482
O.niloticus I S R R Q L I N N L A K H V L F T A N K S S A I M S T H I V 482
M.zebra I S R R Q L I N N L A K H V L F T A N K S S A I M S T H I V 482
G.gallus P Y R R E L I A N L A E H I L F T A N K S C A V M S T H I V 488
M.gallopavo P Y R R E L I A N L A E H I L F T A N K S C A V M S T H I V 488
H.sapiens S L R R R L I A N L A E H I L F T A S K S C A I M S T H I V 488
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R.norvegicus A F R R R L I A N L A E H I L F T A S K S C A I M S T H I V 488
B.taurus S - R R R L I A H L A E H I L F T A S K S C A I M S T H I V 488
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G.aculeatus A C L L L Y R H R Q G V V L S K L V E D F F N M K E E I L S 514
T.rubripes A C L M L Y R H R K G V V L S K L V E D F F S M K E E I L S 513
P.nyererei A C L L L Y R H R Q G V V L S K L V E D F F N M K E E I L S 512
O.niloticus A C L L L Y R H R Q G V V L S K L V E D F F N M K E E I L S 512
M.zebra A C L L L Y R H R Q G V V L S K L V E D F F N M K E E I L S 512
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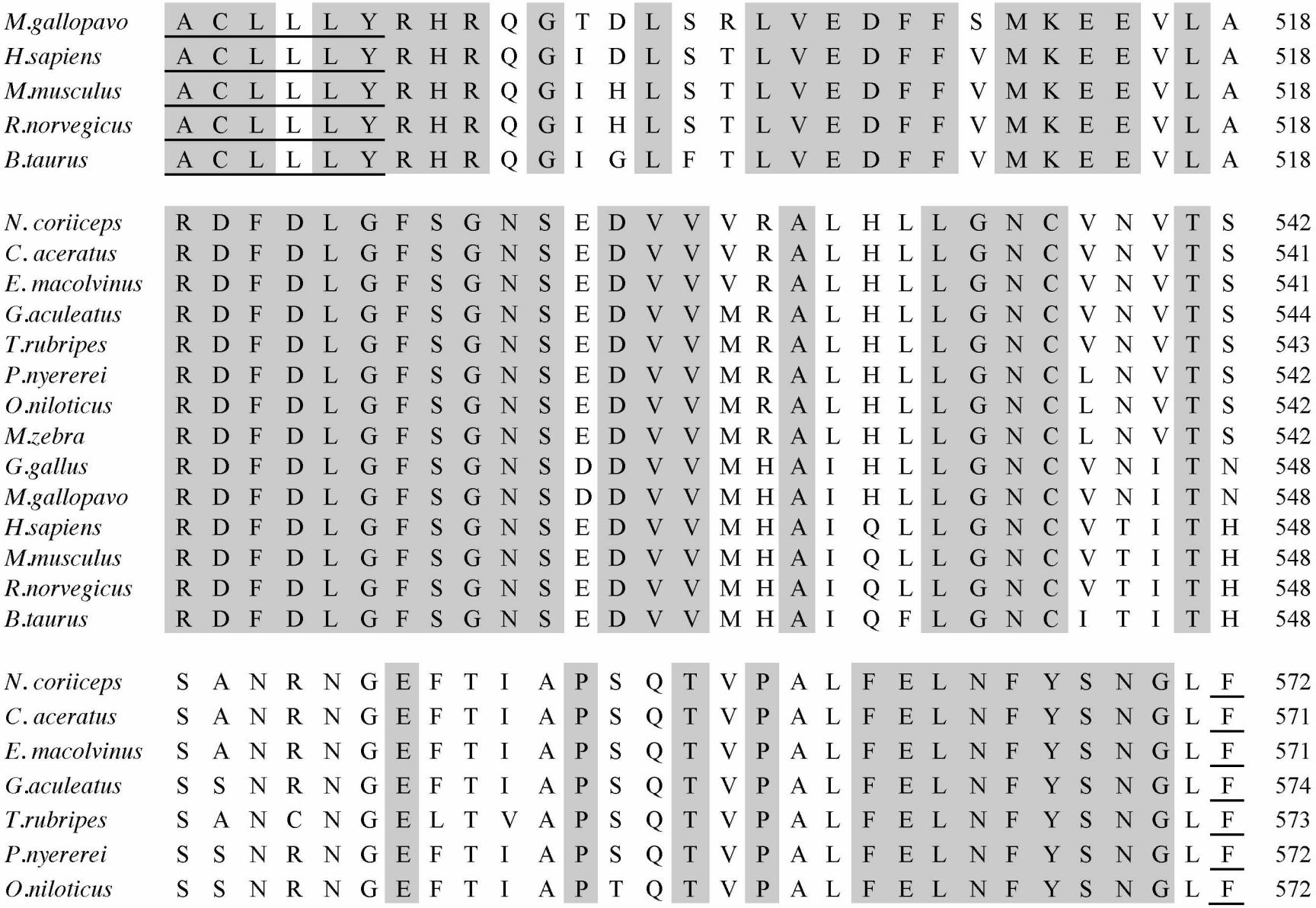
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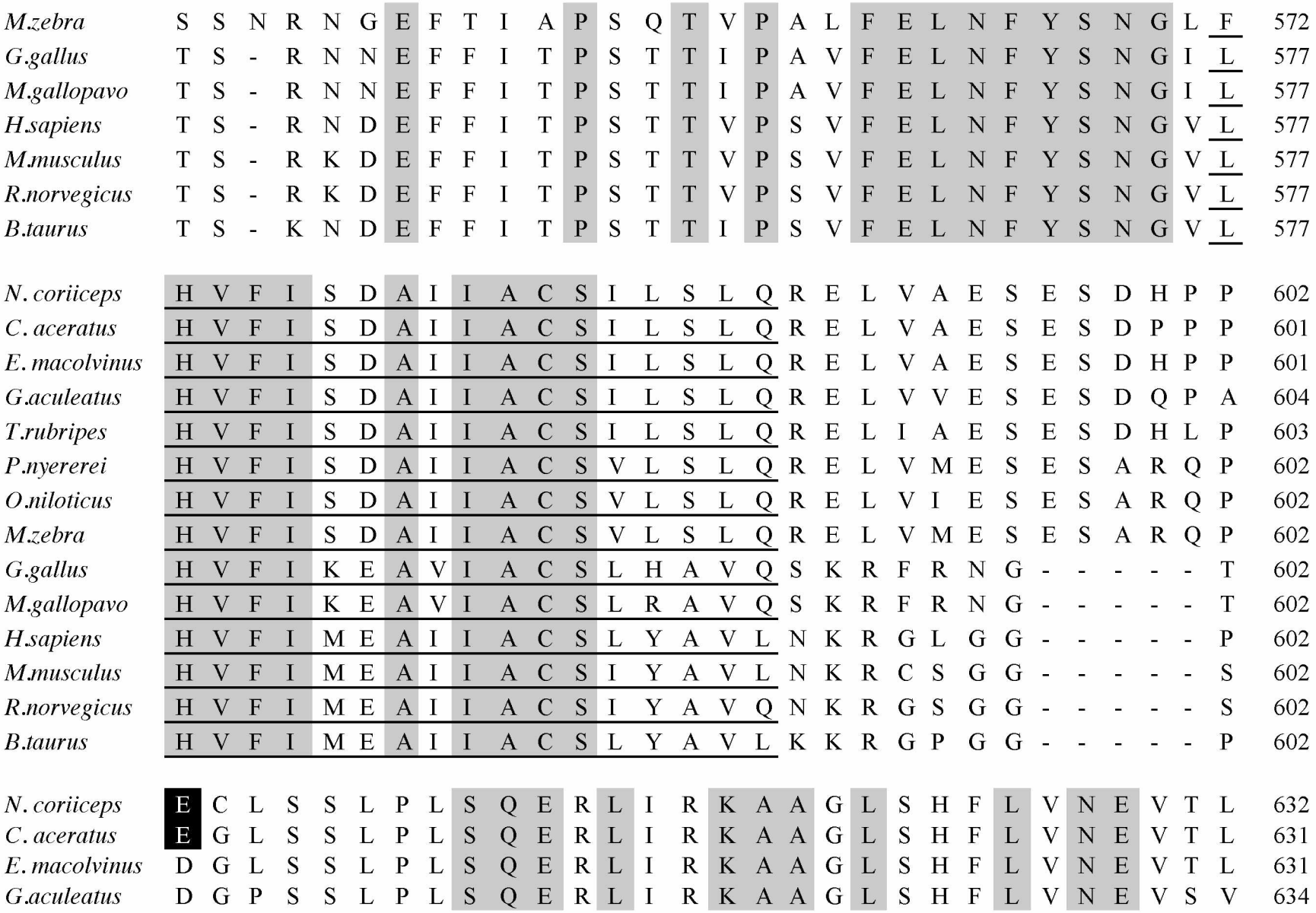
M.musculus A C L L L Y R H R Q G I H L S T L V E D F F V M K E E V L A 518
R.norvegicus A C L L L Y R H R Q G I H L S T L V E D F F V M K E E V L A 518
B.taurus A C L L L Y R H R Q G I G L F T L V E D F F V M K E E V L A 518
N. coriiceps R D F D L G F S G N S E D V V V R A L H L L G N C V N V T S 542
C. aceratus R D F D L G F S G N S E D V V V R A L H L L G N C V N V T S 541
E. macolvinus R D F D L G F S G N S E D V V V R A L H L L G N C V N V T S 541
G.aculeatus R D F D L G F S G N S E D V V M R A L H L L G N C V N V T S 544
T.rubripes R D F D L G F S G N S E D V V M R A L H L L G N C V N V T S 543
P.nyererei R D F D L G F S G N S E D V V M R A L H L L G N C L N V T S 542
O.niloticus R D F D L G F S G N S E D V V M R A L H L L G N C L N V T S 542
M.zebra R D F D L G F S G N S E D V V M R A L H L L G N C L N V T S 542
G.gallus R D F D L G F S G N S D D V V M H A I H L L G N C V N I T N 548
M.gallopavo R D F D L G F S G N S D D V V M H A I H L L G N C V N I T N 548
H.sapiens R D F D L G F S G N S E D V V M H A I Q L L G N C V T I T H 548
M.musculus R D F D L G F S G N S E D V V M H A I Q L L G N C V T I T H 548
R.norvegicus R D F D L G F S G N S E D V V M H A I Q L L G N C V T I T H 548
B.taurus R D F D L G F S G N S E D V V M H A I Q F L G N C I T I T H 548
N. coriiceps S A N R N G E F T I A P S Q T V P A L F E L N F Y S N G L F 572
C. aceratus S A N R N G E F T I A P S Q T V P A L F E L N F Y S N G L F 571
E. macolvinus S A N R N G E F T I A P S Q T V P A L F E L N F Y S N G L F 571
G.aculeatus S S N R N G E F T I A P S Q T V P A L F E L N F Y S N G L F 574
T.rubripes S A N C N G E L T V A P S Q T V P A L F E L N F Y S N G L F 573
P.nyererei S S N R N G E F T I A P S Q T V P A L F E L N F Y S N G L F 572
O.niloticus S S N R N G E F T I A P T Q T V P A L F E L N F Y S N G L F 572


M.zebra S S N R N G E F T I A P S Q T V P A L F E L N F Y S N G L F 572
G.gallus T S - R N N E F F I T P S T T I P A V F E L N F Y S N G I L 577
M.gallopavo T S - R N N E F F I T P S T T I P A V F E L N F Y S N G I L 577
H.sapiens T S - R N D E F F I T P S T T V P S V F E L N F Y S N G V L 577
M.musculus T S - R K D E F F I T P S T T V P S V F E L N F Y S N G V L 577
R.norvegicus T S - R K D E F F I T P S T T V P S V F E L N F Y S N G V L 577
B.taurus T S - K N D E F F I T P S T T I P S V F E L N F Y S N G V L 577
N. coriiceps H V F I S D A I I A C S I L S L Q R E L V A E S E S D H P P 602
C. aceratus H V F I S D A I I A C S I L S L Q R E L V A E S E S D P P P 601
E. macolvinus H V F I S D A I I A C S I L S L Q R E L V A E S E S D H P P 601
G.aculeatus H V F I S D A I I A C S I L S L Q R E L V V E S E S D Q P A 604
T.rubripes H V F I S D A I I A C S I L S L Q R E L I A E S E S D H L P 603
P.nyererei H V F I S D A I I A C S V L S L Q R E L V M E S E S A R Q P 602
O.niloticus H V F I S D A I I A C S V L S L Q R E L V I E S E S A R Q P 602
M.zebra H V F I S D A I I A C S V L S L Q R E L V M E S E S A R Q P 602
G.gallus H V F I K E A V I A C S L H A V Q S K R F R N G - - - - - T 602
M.gallopavo H V F I K E A V I A C S L R A V Q S K R F R N G - - - - - T 602
H.sapiens H V F I M E A I I A C S L Y A V L N K R G L G G - - - - - P 602
M.musculus H V F I M E A I I A C S I Y A V L N K R C S G G - - - - - S 602
R.norvegicus H V F I M E A I I A C S I Y A V Q N K R G S G G - - - - - S 602
B.taurus H V F I M E A I I A C S L Y A V L K K R G P G G - - - - - P 602
N. coriiceps E C L S S L P L S Q E R L I R K A A G L S H F L V N E V T L 632
C. aceratus E G L S S L P L S Q E R L I R K A A G L S H F L V N E V T L 631
E. macolvinus D G L S S L P L S Q E R L I R K A A G L S H F L V N E V T L 631
G.aculeatus D G P S S L P L S Q E R L I R K A A G L S H F L V N E V S V 634

T.rubripes	A	D	I	N	S	L	P	L	S	Q	E	R	L
P.nyererei	G	N	P	S	S	H	L	L	S	Q	E	R	L
O.niloticus	G	N	P	S	N	L	L	L	S	Q	E	R	L
M.zebra	G	N	P	S	S	H	L	L	S	Q	E	R	L
G.gallus	N	G	A	S	P	S	L	I	S	Q	E	H	L
M.gallopavo	N	G	A	S	P	S	L	I	S	Q	E	H	L
H.sapiens	T	S	T	P	P	N	L	I	S	Q	E	Q	L
M.musculus	A	G	G	L	G	N	L	I	S	Q	E	Q	L
R.norvegicus	A	G	G	L	G	N	L	I	S	Q	E	Q	L
B.taurus	A	S	-	-	P	S	L	V	S	Q	E	Q	L

N. coriiceps	A	P	P	C	Q	T	I	Y	Q	V	F	H	D
C. aceratus	A	P	P	C	Q	T	I	Y	Q	V	F	H	D
E. macolvinus	A	P	P	C	Q	T	I	Y	Q	V	F	H	D
G.aculeatus	A	P	P	C	Q	T	I	Y	Q	V	F	H	D
T.rubripes	A	P	P	C	Q	T	L	Y	Q	V	F	H	D
P.nyererei	A	P	P	C	Q	T	I	Y	Q	V	F	H	D
O.niloticus	A	P	P	C	Q	T	I	Y	Q	V	F	H	D
M.zebra	A	P	P	C	Q	T	I	Y	Q	V	F	H	D
G.gallus	S	L	P	C	Q	L	I	Y	Q	V	C	H	E
M.gallopavo	S	L	P	C	Q	L	I	Y	Q	V	C	H	E
H.sapiens	S	L	P	C	Q	T	F	Y	Q	V	C	H	E
M.musculus	S	L	P	C	Q	T	F	Y	Q	V	C	H	E
R.norvegicus	S	L	P	C	Q	T	F	Y	Q	V	C	Q	E
B.taurus	S	L	P	C	Q	T	F	Y	Q	I	C	H	E

N. coriiceps	D	Q	E	E	L	S	P	S	P	T	D	E	P
C. aceratus	D	Q	E	E	L	S	P	S	P	T	E	E	P
E. macolvinus	D	Q	E	E	L	S	P	S	P	T	E	E	P
G.aculeatus	D	H	E	E	L	S	P	S	P	T	E	E	P

I	R	K	A	A	G	L	S	Y	F	L	T	N	E	V	M	V	633
I	R	K	A	A	G	L	S	H	F	L	I	N	E	V	A	V	632
I	R	K	A	A	G	L	S	H	F	L	I	N	E	V	A	V	632
I	R	K	A	A	G	L	S	H	F	L	I	N	E	V	A	V	632
V	R	K	A	A	S	L	C	Y	L	L	S	N	E	F	T	V	632
V	R	K	A	A	S	L	C	Y	L	L	S	N	E	F	T	V	632
V	R	K	A	A	S	L	C	Y	L	L	S	N	E	G	T	I	632
V	R	K	A	A	S	L	C	Y	L	L	S	N	E	G	T	I	632
V	R	K	A	A	S	L	C	Y	L	L	S	N	E	G	T	I	632
V	H	K	A	A	S	L	C	Y	L	L	S	N	E	G	T	I	632

A	V	A	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	661
A	I	A	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	660
A	V	T	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	660
A	V	T	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	663
A	V	T	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	662
T	V	T	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	661
T	V	T	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	661
T	V	T	R	L	I	Q	Y	G	V	L	Y	V	A	E	-	E	661
A	V	E	K	L	I	Q	Y	G	I	L	L	V	A	E	-	D	661
A	V	E	K	L	I	Q	Y	G	I	L	L	V	A	E	Q	D	662
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T	V	G	R	F	I	Q	Y	G	I	L	I	V	A	E	Q	D	662

W	P	K	K	F	P	E	P	L	S	W	R	S	D	E	E	D	691
W	P	K	K	F	P	E	P	L	S	W	R	S	D	E	E	D	690
W	P	K	K	F	P	E	P	L	S	W	R	S	D	E	E	D	690
W	P	K	K	F	P	E	P	L	S	W	R	S	D	E	E	D	693

T.rubripes	D	Q	E	E	L	S	P	S	P	T	E
P.nyererei	D	Q	E	E	L	S	P	S	P	T	D
O.niloticus	D	Q	E	E	L	S	P	S	P	T	D
M.zebra	D	Q	E	E	L	S	P	S	P	T	D
G.gallus	D	Q	E	D	V	S	P	S	L	T	E
M.gallopavo	D	Q	E	D	V	S	P	S	L	T	E
H.sapiens	D	Q	E	D	I	S	P	S	L	A	E
M.musculus	D	Q	E	D	V	S	P	G	L	A	E
R.norvegicus	D	Q	E	D	V	S	P	G	L	A	E
B.taurus	D	Q	E	D	I	S	P	G	L	A	E

N. coriiceps	E	D	S	D	F	G	E	E	Q	R	D
C. aceratus	E	D	S	D	F	G	E	E	Q	R	D
E. macolvinus	E	D	S	D	F	G	E	E	Q	R	D
G.aculeatus	E	D	S	D	F	G	E	E	Q	R	D
T.rubripes	E	D	S	D	F	G	E	E	Q	R	D
P.nyererei	E	D	S	D	F	G	E	E	Q	R	D
O.niloticus	E	D	S	D	F	G	E	E	Q	R	D
M.zebra	E	D	S	D	F	G	E	E	Q	R	D
G.gallus	E	D	S	D	F	G	E	E	Q	R	D
M.gallopavo	E	D	S	D	F	G	E	E	Q	R	D
H.sapiens	E	D	S	D	F	G	E	E	Q	R	D
M.musculus	E	D	S	D	F	G	E	E	Q	R	D
R.norvegicus	E	D	S	D	F	G	E	E	Q	R	D
B.taurus	E	D	S	D	F	G	E	E	Q	R	D

N. coriiceps	R	L	V	S	P	V	L	E	A	Y	S
C. aceratus	R	L	V	S	P	V	L	E	A	Y	S
E. macolvinus	R	L	V	S	P	V	L	E	A	Y	S
G.aculeatus	R	L	V	S	P	V	L	E	A	Y	S

E	P	W	P	K	K	F	P	E	P	L	S	W	R	S	D	E	E	D	692
E	P	W	P	K	K	F	A	E	P	L	S	W	R	S	D	E	E	D	691
E	P	W	P	K	K	F	A	E	P	L	S	W	R	S	D	E	E	D	691
E	P	W	P	K	K	F	A	E	P	L	S	W	R	S	D	E	E	D	691
Q	Q	W	N	K	K	L	P	E	P	L	T	W	R	S	D	E	E	D	691
Q	Q	W	N	K	K	L	P	E	P	L	T	W	R	S	D	E	E	D	692
Q	Q	W	D	K	K	L	P	E	P	L	S	W	R	S	D	E	E	D	692
Q	Q	W	D	K	K	L	P	E	-	L	N	W	R	S	D	E	E	D	692
Q	Q	W	N	K	K	L	P	E	P	L	N	W	R	S	D	E	E	D	692
Q	Q	W	D	K	K	L	P	E	P	L	S	W	R	S	D	E	E	D	692

R	Y	L	K	V	S	V	S	A	E	H	Q	E	F	F	V	F	L	Q	721
R	Y	L	K	V	S	V	S	A	E	H	Q	E	F	F	V	F	L	Q	720
R	Y	L	K	V	S	V	S	A	E	H	Q	E	F	F	V	F	L	Q	720
R	Y	L	K	V	S	L	S	A	E	H	Q	E	F	F	V	F	L	Q	723
R	Y	L	K	V	S	I	S	A	E	H	Q	D	F	F	I	F	L	Q	722
R	Y	L	K	V	S	V	S	A	E	H	Q	E	F	F	V	F	L	Q	721
R	Y	L	K	V	S	V	S	A	E	H	Q	E	F	F	V	F	L	Q	721
R	Y	L	K	V	S	V	S	A	E	H	Q	E	F	F	V	F	L	Q	721
C	Y	L	K	V	S	Q	S	Q	E	H	Q	Q	Y	I	T	F	L	Q	721
C	Y	L	K	V	S	Q	S	Q	E	H	Q	Q	Y	I	T	F	L	Q	722
C	Y	L	K	V	S	Q	S	K	E	H	Q	Q	F	I	T	F	L	Q	722
C	Y	L	K	V	S	Q	S	K	E	H	Q	Q	F	I	T	F	L	Q	722
C	Y	L	K	V	S	Q	A	K	E	H	Q	Q	F	I	T	F	L	Q	722
C	Y	L	K	V	S	Q	S	K	E	H	Q	Q	F	I	T	F	L	Q	722

G	A	A	I	F	V	H	S	L	S	Q	P	M	A	E	S	D	Y	T	751
G	A	A	I	F	V	H	S	L	S	Q	P	M	A	E	S	D	Y	T	750
G	A	A	I	F	V	H	S	L	S	Q	P	M	A	E	S	D	Y	T	750
G	A	A	I	F	V	H	S	L	S	Q	P	M	A	E	S	E	Y	T	753

T.rubripes	R	L	V	S	P	V	L	E	A	Y	S	G	A	A	I	F	I	H	S	L	V	Q	P	T	S	E	S	D	Y	T	752
P.nyererei	R	L	L	S	P	V	L	E	A	Y	S	G	A	A	I	F	V	H	S	L	S	Q	P	M	V	E	S	D	Y	T	751
O.niloticus	R	L	L	S	P	V	L	E	A	Y	S	G	A	A	I	F	V	H	S	L	S	Q	P	M	V	E	S	D	Y	T	751
M.zebra	R	L	L	S	P	V	L	E	A	Y	S	G	A	A	I	F	V	H	S	L	S	Q	P	M	V	E	S	D	Y	T	751
G.gallus	R	L	L	G	P	L	L	E	A	Y	S	S	A	V	I	F	V	H	N	F	S	G	P	V	S	E	S	E	Y	L	751
M.gallopavo	R	L	L	G	P	L	L	E	A	Y	S	S	A	V	I	F	V	H	N	F	S	G	P	V	S	E	S	E	Y	L	752
H.sapiens	R	L	L	G	P	L	L	E	A	Y	S	S	A	A	I	F	V	H	N	F	S	G	P	V	P	E	P	E	Y	L	752
M.musculus	R	L	L	G	P	L	L	E	A	Y	S	S	A	A	I	F	V	H	N	F	S	G	P	V	P	E	S	E	Y	L	752
R.norvegicus	R	L	L	G	P	L	L	E	A	Y	S	S	A	A	I	F	V	H	T	F	R	G	P	V	P	E	S	E	Y	L	752
B.taurus	R	L	L	G	P	L	L	E	A	Y	S	S	A	A	V	F	I	H	N	F	G	G	P	V	P	E	P	E	F	L	752
N. coriiceps	Q	R	L	F	R	Y	L	L	T	R	T	E	R	G	V	A	A	Y	-	-	-	-	-	-	-	-	-	-	-	-	769
C. aceratus	Q	R	L	F	R	Y	L	L	T	R	T	E	R	G	V	A	A	Y	-	-	-	-	-	-	-	-	-	-	-	-	768
E. macolvinus	Q	R	L	F	R	Y	L	L	T	R	T	E	R	G	V	A	A	Y	-	-	-	-	-	-	-	-	-	-	-	-	768
G.aculeatus	Q	R	L	F	R	Y	L	L	T	R	T	E	R	G	V	A	A	Y	-	-	-	-	-	-	-	-	-	-	-	-	771
T.rubripes	Q	K	L	F	R	Y	L	L	T	R	T	E	K	G	L	A	V	Y	-	-	-	-	-	-	-	-	-	-	-	-	770
P.nyererei	Q	R	L	F	R	Y	L	L	T	R	T	E	R	G	V	A	G	Y	-	-	-	-	-	-	-	-	-	-	-	-	769
O.niloticus	Q	R	L	F	R	Y	L	L	T	R	T	E	R	G	V	A	G	Y	-	-	-	-	-	-	-	-	-	-	-	-	769
M.zebra	Q	R	L	F	R	Y	L	L	T	R	T	E	R	G	V	A	G	Y	-	-	-	-	-	-	-	-	-	-	-	-	769
G.gallus	Q	K	L	H	R	H	L	I	N	R	T	E	K	N	V	A	V	Y	G	M	S	N	S	L	S	S	V	L	Y	L	781
M.gallopavo	Q	K	L	H	R	H	L	I	S	R	T	E	K	N	V	A	V	Y	-	-	-	-	-	-	-	-	-	-	-	-	770
H.sapiens	Q	K	L	H	K	Y	L	I	T	R	T	E	R	N	V	A	V	Y	-	-	-	-	-	-	-	-	-	-	-	-	770
M.musculus	Q	K	L	H	R	Y	L	I	T	R	T	E	R	N	V	A	V	Y	-	-	-	-	-	-	-	-	-	-	-	-	770
R.norvegicus	Q	K	L	H	R	Y	L	L	T	R	T	E	R	N	V	A	V	Y	-	-	-	-	-	-	-	-	-	-	-	-	770
B.taurus	Q	K	L	H	K	Y	L	I	T	R	T	E	R	R	V	A	V	Y	-	-	-	-	-	-	-	-	-	-	-	-	770
N. coriiceps	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	773
C. aceratus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	772
E. macolvinus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	772
G.aculeatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	775

T.rubripes	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	774
P.nyererei	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	773
O.niloticus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	773
M.zebra	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	E	S	A	773
G.gallus	I	L	C	V	P	P	G	S	N	I	A	Q	I	Q	L	Q	F	V	Y	I	C	V	F	F	L	A	A	E	S	A	811
M.gallopavo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	E	S	A	774
H.sapiens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	E	S	A	774
M.musculus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	E	S	A	774
R.norvegicus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	E	S	A	774
B.taurus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	E	S	A	774

N. coriiceps	T	H	Y	L	V	K	N	T	V	R	T	F	K	E	L	G	V	L	K	Q	R	K	E	N	Q	V	T	T	L	E	803
C. aceratus	T	H	Y	L	V	K	N	T	V	R	T	F	R	E	L	G	V	L	K	Q	R	K	E	N	Q	V	T	T	L	E	802
E. macolvinus	T	H	Y	L	V	K	N	T	V	R	T	F	K	E	L	G	V	L	K	Q	R	R	E	N	Q	V	T	T	L	E	802
G.aculeatus	T	H	Y	L	V	K	N	T	V	K	T	F	K	E	L	G	V	L	K	Q	R	K	E	N	Q	V	T	A	V	E	805
T.rubripes	T	H	Y	L	V	K	N	T	V	R	T	F	K	E	L	G	V	L	K	V	R	K	E	N	K	M	T	I	L	E	804
P.nyererei	T	H	Y	L	V	K	N	T	V	R	T	F	K	E	L	G	V	L	K	E	R	R	E	N	K	V	T	T	L	E	803
O.niloticus	T	H	Y	L	V	K	N	T	V	R	T	F	K	E	L	G	V	L	K	E	R	R	E	N	K	V	T	T	L	E	803
M.zebra	T	H	Y	L	V	K	N	T	V	R	T	F	K	E	L	G	V	L	K	E	R	R	E	N	K	V	T	T	L	E	803
G.gallus	T	Y	S	H	V	K	N	A	V	K	V	F	K	E	I	G	V	F	I	Q	T	N	Q	K	R	D	T	I	L	E	841
M.gallopavo	T	Y	S	H	V	K	N	A	V	K	V	F	K	E	I	G	V	F	S	Q	T	N	Q	K	K	D	T	I	L	E	804
H.sapiens	T	Y	C	L	V	K	N	A	V	K	M	F	K	D	I	G	V	F	K	E	T	K	Q	K	R	V	S	V	L	E	804
M.musculus	T	Y	C	L	V	K	N	A	V	K	M	F	K	D	I	G	V	F	K	E	T	K	Q	K	R	V	S	V	L	E	804
R.norvegicus	T	Y	C	L	V	K	N	A	V	K	M	F	K	D	I	G	V	F	K	E	T	K	Q	K	R	A	S	V	L	E	804
B.taurus	T	Y	C	L	V	K	N	A	V	K	T	F	K	D	I	G	V	F	K	E	T	K	Q	K	R	V	S	G	L	E	804

N. coriiceps	L	S	S	T	F	L	P	Q	A	N	R	N	K	L	L	Q	Y	I	L	G	F	T	L	L						827
C. aceratus	L	S	S	T	F	L	P	Q	A	N	R	N	K	L	L	Q	Y	I	L	G	F	A	L	L						826
E. macolvinus	L	S	S	T	F	L	P	Q	A	N	R	N	K	L	L	Q	Y	I	L	G	F	T	L	L						826
G.aculeatus	L	S	S	T	F	L	P	Q	A	N	R	N	K	L	L	H	Y	I	L	G	F	A	L	L						829

T.rubripes	L	S	S	T	F	L	P	Q	A	N	R	N	K	L	L	Q	Y	I	L	G	F	T	L	L	828
P.nyererei	L	S	S	T	F	L	P	Q	E	N	R	N	K	L	L	Q	Y	I	L	G	F	T	L	L	827
O.niloticus	L	S	S	T	F	L	P	Q	E	N	R	N	K	L	L	Q	Y	I	L	G	F	T	L	L	827
M.zebra	L	S	S	T	F	L	P	Q	E	N	R	N	K	L	L	Q	Y	I	L	G	F	T	L	L	827
G.gallus	L	S	T	T	F	L	P	Q	R	N	R	Q	K	L	L	E	F	I	M	S	F	M	V	L	865
M.gallopavo	L	S	T	T	F	L	P	Q	R	N	R	Q	K	L	L	E	F	I	M	S	F	M	V	L	828
H.sapiens	L	S	S	T	F	L	P	Q	C	N	R	Q	K	L	L	E	Y	I	L	S	F	V	V	L	828
M.musculus	L	S	S	T	F	L	P	Q	C	N	R	Q	K	L	L	E	Y	I	L	S	F	V	V	L	828
R.norvegicus	L	S	T	T	F	L	P	Q	G	S	R	Q	K	L	L	E	Y	I	L	S	F	V	V	L	828
B.taurus	L	S	N	T	F	L	P	Q	C	N	R	Q	K	L	L	E	Y	I	L	S	L	V	V	L	828

Figure 1.4. (pages 42-57) Amino acid sequence alignment of fish, bird, and mammalian GPAT1. Identical residues are shaded, active site motifs I-IV are boxed in solid bold, transmembrane domains I and II are underlined, and substitutions unique to Antarctic notothenioids are shown in white lettering on black.

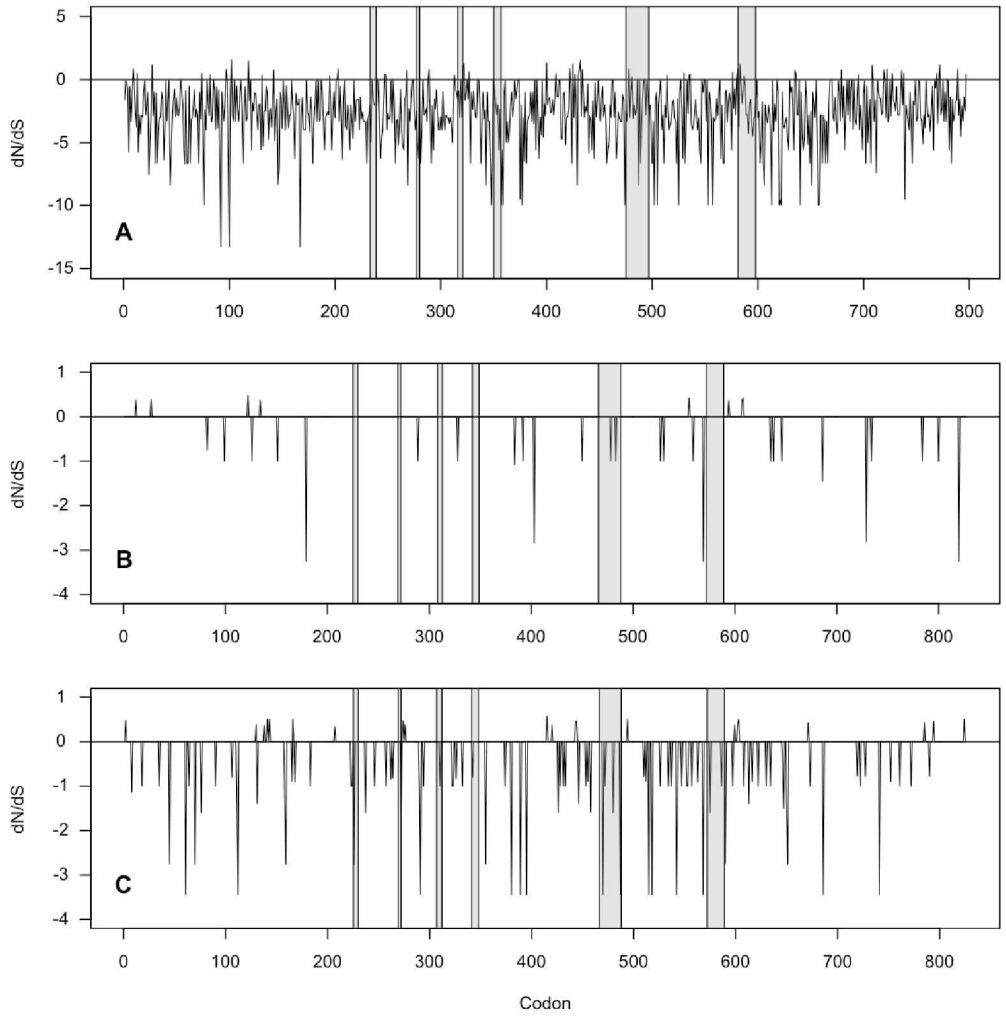

Figure 1.5. dN/dS ratios in GPAT1 of selected fish species. Ratio values >1 represent positive selection, values equal to 1 are indicative of neutral selection, and <1 represent negative or purifying selection. Areas of interest in GPAT1 of fishes are marked by grey bars (from left to right: the first four bars are motifs I, II, III, and IV of the active site, the last two are transmembrane domains I and II). Species included in this data are: **Panel A** *Notothenia coriiceps, Cheanocephalus aceratus, Eleginops maclovinus*, *Gasteosteus aculeatus, Takifugu rubripes, Esox Lucius, Maylandia zebra, Oreochromis niloticus, Pundamilia nyererei, Cynoglossus semilaevis*, and *Gadus. Morhua* **Panel B** (Cichlids) *Maylandia zebra, Oreochromis. niloticus,* and *Pundamilia nyererei* **Panel C** (Notothenioids) *Notothenia coriiceps, Cheanocephalus aceratus*, and *Eleginops maclovinus*.

1.8 TABLES

Table 1.1
Gene-specific primers used to quantify relative transcript levels. Primers used for measuring mRNA of GPAT1, GPAT2, TBP, EF1-α, and 18S rRNA in heart ventricle of *N. coriiceps* and *C. aceratus*

Primer name	Nucleotide sequence
GPAT1	F 5' AGAGCCCCTTTCCTGGAGAA 3'
	R 5' TCTGCGGAAACGCTCACC 3'
GPAT2	F 5' GGCTTCCACAACCTGCTGAG 3'
	R 5' GCCTCGATACCTGGTGTGTGT 3'
TBP	F 5' CGGGAGCCAAGAGTGAGGA 3'
	R 5' GAGCGTATTTTCTGGCAGCTAAC 3'
EF1-α	F 5' CTGGAAGCCAGTGAAAAGATGAC 3'
	R 5' ACCACATCCAAGGAAGGCAG 3'
18S	F 5' ACCACATCCAAGGAAGGCAG 3'
	R 5' CCGAGTCGGGAGTGGGTAAT 3'

F indicates forward, R indicates reverse

Table 1.2

Degenerate and gene-specific primers used to obtain GPAT1 and GPAT2 cDNA. Sequences were obtained in *N.coriiceps*, *C. aceratus* and *E. maclovinus* for GPAT1 and partial sequences were obtained in *N. coriiceps* and *C. aceratus* for GPAT2

Primer name	Type	Nucleotide sequence (5'-3')
GPAT1 F	Degenerate	GCCTGCGGAACGTGAT**H**TA**Y**AT**H**AA
GPAT1 R	Degenerate	TGAGGGGCCTTGATGTT**R**TG**R**CA**R**AA
GPAT1 F2	Specific	TCGACTACTTGCTCATCACGTT
GPAT1 R2	Degenerate	TCACGGCGTCGTGGAA**N**AC**Y**TG**R**TA
GPAT2 F	Degenerate	GGGCCAGTGCTGCCA**Y**CA**R**TG**Y**AC
GPAT2 R	Degenerate	CGGTGCCGGGTGGA**Y**GA**N**TGG**Y**T
GPAT1 5' RACE	Specific	GAGGAGTTGTTGCGTCAGCAGCAGTTTC
GPAT1 3' RACE	Specific	AGCGCTAACCGCAACGGAGAGTTC
GPAT1 Emac F	Specific	CAGTCAGGAGAGACTCATCAGAAAGG
GPAT1 Emac R	Specific	CTGGGATTGTTTAGAAGTAACCAGTA
UPM	Specific	CTAATACGACTCACTATAGGGC
M13 F	Specific	GTAAAACGACGGCCAG
M13 R	Specific	CAGGAAACAGCTATGAC

F indicates forward, R indicates reverse, UPM indicates Universal Primer Mix

Degenerate nucleotides are indicated as H, N, R, and Y (H=A,C,T; N=A,C,G,T; R=A,G; Y=C,T)

Table 1.3

Housekeeping gene analysis. Results from Bestkeeper and 2-way ANOVA

Gene	SD	*r*	*p*-value for *r*	*F*-value for ANOVA
Liver				
18S	0.19	0.306	0.362	
EF1-α	0.53	0.932	0.001	
TBP	0.55	0.806	0.003	
Ventricle				
18S	0.42	0.959	0.001	
EF1-α	0.58	0.909	0.001	
TBP	0.59	0.808	0.001	
Liver and Ventricle				
18S	0.7	0.925	0.001	1.003
EF1-α	0.99	0.966	0.001	1.003
TBP	0.57	0.378	0.075	0.002

r, Pearson's correlation coefficient with Bestkeeper index.

N=24; six individuals per tissue for *N. corriceps* and *C. acerartus*

Table 1.4
Active site motifs of GPAT1

		I						II				III						IV							
	N.coriiceps	H	K	S	H	I	D	F	F	I	R	Y	L	E	G	T	R	V	L	V	V	P	V	G	I
A	*C.aceratus*	H	K	S	H	I	D	F	F	I	R	Y	L	E	G	T	R	V	L	V	V	P	V	G	I
	E.maclovinus	H	K	S	H	I	D	F	F	I	R	Y	L	E	G	T	R	V	L	V	V	P	V	G	I
	G.aculeatus	H	K	S	H	I	D	F	F	I	R	Y	L	E	G	T	R	V	L	V	V	P	V	G	I
	T.rubripes	H	K	S	H	I	D	F	F	I	R	Y	L	E	G	T	R	V	L	V	V	P	V	G	I
B	*P.nyererei*	H	K	S	H	I	D	F	F	I	R	F	L	E	G	T	R	V	L	V	V	P	V	G	I
	O.niloticus	H	K	S	H	I	D	F	F	I	R	F	L	E	G	T	R	V	L	V	V	P	V	G	I
	M.zebra	H	K	S	H	I	D	F	F	I	R	F	L	E	G	T	R	V	L	V	V	P	V	G	I
	M.gallopavo	H	K	S	H	I	D	F	F	I	R	F	L	E	G	T	R	V	L	I	I	P	V	G	I
	G.gallus	H	K	S	H	I	D	F	F	I	R	F	L	E	G	T	R	V	L	I	I	P	V	G	I
C	*H.sapiens*	H	R	S	H	I	D	F	F	I	R	F	L	E	G	T	R	I	L	I	I	P	V	G	I
	M.musculus	H	R	S	H	I	D	F	F	I	R	F	L	E	G	T	R	I	L	V	I	P	V	G	I
	R.norvegicus	H	R	S	H	I	D	F	F	I	R	F	L	E	G	T	R	I	L	V	I	P	V	G	I
	B.taurus	H	R	S	H	I	D	F	F	I	R	F	L	E	G	T	R	I	L	I	I	P	G	G	I

Alignment of active site motifs I, II, III and IV of GPAT1: **A** Notothenioids **B** temperate and tropical fishes **C** birds and mammals. Sequences were obtained from databases or from this study by sequencing. Shaded columns indicate substitutions of amino acids between vertebrate taxa.

Table 1.5A

Transmembrane domain I of GPAT1

		TMD I																					
A	*N.coriiceps*	V	L	F	T	A	N	K	S	S	A	I	M	S	T	H	I	I	A	C	L	L	L	Y
	C.aceratus	V	L	F	T	A	N	K	S	S	A	I	M	S	T	H	I	I	A	C	L	L	L	Y
	E.maclovinus	V	L	F	T	A	N	K	S	S	A	I	M	S	T	H	I	I	A	C	L	L	L	Y
B	*G.aculeatus*	V	L	F	T	A	N	K	S	S	A	I	M	S	T	H	I	V	A	C	L	L	L	Y
	T.rubpies	V	I	F	T	A	N	K	S	S	A	I	M	S	T	H	I	V	A	C	L	M	L	Y
	P.nyererei	V	L	F	T	A	N	K	S	S	A	I	M	S	T	H	I	V	A	C	L	L	L	Y
	O.niloticus	V	L	F	T	A	N	K	S	S	A	I	M	S	T	H	I	V	A	C	L	L	L	Y
	M.zebra	V	L	F	T	A	N	K	S	S	A	I	M	S	T	H	I	V	A	C	L	L	L	Y
C	*M.gallopavo*	I	L	F	T	A	N	K	S	C	A	V	M	S	T	H	I	V	A	C	L	L	L	Y
	G.gallus	I	L	F	T	A	N	K	S	C	A	V	M	S	T	H	I	V	A	C	L	L	L	Y
	H.sapiens	I	L	F	T	A	S	K	S	C	A	I	M	S	T	H	I	V	A	C	L	L	L	Y
	M.musculus	I	L	F	T	A	S	K	S	C	A	I	M	S	T	H	I	V	A	C	L	L	L	Y
	R.norvegicus	I	L	F	T	A	S	K	S	C	A	I	M	S	T	H	I	V	A	C	L	L	L	Y
	B.taurus	I	L	F	T	A	S	K	S	C	A	I	M	S	T	H	I	V	A	C	L	L	L	Y

Alignment of GPAT1 transmembrane domain I: **A** Notothenioids **B** temperate and tropical fishes **C** birds and mammals. Sequences were obtained from databases or from this study by sequencing. TMDI; transmenbrane domain I. Shaded columns indicate substitutions of amino acids between vertebrate taxa.

Table 1.5B
Transmembrane domain II of GPAT1

		TMD II																
A	*N.coriiceps_GPAT1*	F	H	V	F	I	S	D	A	I	I	A	C	S	I	L	S	L	Q
	C.aceratus_GPAT1	F	H	V	F	I	S	D	A	I	I	A	C	S	I	L	S	L	Q
	E.maclovinus_GPAT1	F	H	V	F	I	S	D	A	I	I	A	C	S	I	L	S	L	Q
B	*G.aculeatus_GPAT1*	F	H	V	F	I	S	D	A	I	I	A	C	S	I	L	S	L	Q
	T.rubripes	F	H	V	F	I	S	D	A	I	I	A	C	S	I	L	S	L	Q
	P.nyererei_GPAT1	F	H	V	F	I	S	D	A	I	I	A	C	S	V	L	S	L	Q
	O.niloticus_GPAT1	F	H	V	F	I	S	D	A	I	I	A	C	S	V	L	S	L	Q
	M.zebra_GPAT1	F	H	V	F	I	S	D	A	I	I	A	C	S	V	L	S	L	Q
C	*M.gallopavo_GPAT1*	L	H	V	F	I	K	E	A	V	I	A	C	S	L	R	A	V	Q
	G.gallus_GPAT1	L	H	V	F	I	K	E	A	V	I	A	C	S	L	H	A	V	Q
	H.sapiens_GPAT1	L	H	V	F	I	M	E	A	I	I	A	C	S	L	Y	A	V	L
	M.musculus_GPAT1	L	H	V	F	I	M	E	A	I	I	A	C	S	I	Y	A	V	L
	R.norvegicus_GPAT1	L	H	V	F	I	M	E	A	I	I	A	C	S	I	Y	A	V	Q
	B.taurus_GPAT1	L	H	V	F	I	M	E	A	I	I	A	C	S	L	Y	A	V	L

Alignment of GPAT1 transmembrane domain II: **A** Notothenioids **B** temperate and tropical fishes **C** birds and mammals. Sequences were obtained from databases or from this study by sequencing. TMDII; transmembrane domain II. Shaded columns indicate substitutions of amino acids between vertebrate taxa.

Table 1.6

Nonsynonymous amino acid substitutions in GPAT1 orthologs

Concensus Amino acid	Ser	Asn	Ser	Met	Cys	Lle	Gln	Asn	His	Val	Leu
Amino acid Position	34	122	138	342	343	421	446	455	610	748	812
Codon	TCG	AAC	AGC	ATG	TGG	ATT	GAG	AAC	CAC	GTC	CTG
N.coriiceps					Tyr						
C.aceratus					Tyr				Pro		
E.maclovinus			Cys		Tyr						
G. aculeatus			Asn		Tyr				Gln		Val
T.rubripes	Leu		Lys	Leu	His			His		Ile	
E.lucius		Gln						Ile	Gly		Val
M.zebra		His				Ser	Asp		Arg		
O.niloticus		His				Ser	Asp		Arg		
P.nyererei		His				Ser	Asp		Arg		
C.semilaevis				Leu		Val			Gln		
G.morhua	Leu	Arg		Leu		Leu	Asn	Ser		Leu	

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CHAPTER 2. Molecular Drivers of Mitochondrial Membrane Proliferation in response to cold acclimation in threespine stickleback [1]

2.1 ABSTRACT

It is unknown how the synthesis of mitochondrial phospholipids is integrated into mitochondrial biogenesis in fish or mammals. Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the addition of fatty acyl CoA to the *sn*-1 position of glycerol-3 phosphate, which is considered the rate-limiting step in phospholipid biosynthesis. Previous studies have shown that mitochondrial volume density increases in oxidative muscle but not liver of *Gasterosteus aculeatus* (threespine stickleback) in response to cold acclimation. We hypothesized that maximal activity of GPAT would increase in oxidative muscle but not liver during cold acclimation, coinciding with mitochondrial biogenesis. GPAT activity was measured in liver and oxidative muscle of threespine stickleback cold-acclimated to 8 °C and warm-acclimated to 20 °C. In addition, mRNA levels of enzymes involved in phospholipid synthesis, including CDP-diacylglycerol synthase-1 (CDS1), CDS2, GPAT1 and 1-acylglycerol-3-phosphate acyltransferase-2 (AGPAT2) were quantified in liver and pectoral of stickleback harvested throughout cold acclimation to 8 °C. GPAT activity increased in response to cold acclimation in pectoral muscle but not liver. However, transcript levels of GPAT1 increased in liver but not pectoral muscle in response to cold acclimation, and transcript levels of AGPAT2 increased in pectoral adductor but not liver. Overall our results suggest that GPAT and/or AGPAT increase during cold acclimation and may contribute to mitochondrial phospholipid biosynthesis required for mitochondrial biogenesis.

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2.2 INTRODUCTION

Mitochondrial density increases in response to cold acclimation in oxidative skeletal muscle of many cold-active teleosts, including goldfish, striped bass, zebrafish and stickleback (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010; Tyler and Sidell, 1984). Mitochondria are chimeric organelles, possessing proteins encoded in both the mitochondrial and nuclear genomes. Consequently, the proliferation of mitochondria through mitochondrial biogenesis requires coordination of the two genomes to synthesize mitochondrial proteins and lipids, and to replicate the mitochondrial genome. In mammals, the co-transcriptional activator PGC-1α is considered the master regulator of mitochondrial biogenesis. It transactivates expression of mitochondrial transcription factor A (TFAM), which is then translated on cytosolic ribosomes and imported into the mitochondrion where it induces mtDNA replication and transcription, leading to an increase in mitochondrial DNA and proteins (Scarpulla, 2008). Much less is known about mitochondrial biogenesis in fishes but several studies suggest that nuclear respiratory factor-1 (NRF-1), rather than PGC-1α, orchestrates the process (Bremer et al., 2012; Orczewska et al., 2010). While mammalian studies have clearly delineated how mtDNA is replicated and mitochondrial proteins are synthesized during mitochondrial biogenesis, the molecular drivers mediating the synthesis of mitochondrial phospholipids remain largely unknown (O'Brien and Mueller, 2010).

Glycerol-3-phosphate acyltransferase (GPAT) is considered the rate-limiting step in triacylglycerol and phospholipid biosynthesis. It catalyzes the addition of fatty acyl CoA to the *sn*-1 position of glycerol–3 phosphate, producing lysophospatidic acid (LPA). There are four isoforms of GPAT, two of which are localized to the outer mitochondrial membrane (GPAT 1 and 2), and two of which are localized to the endoplasmic reticulum (ER) membrane (GPAT 3 and 4) (Bell and Coleman, 1980; Coleman and Lee, 2004). Downstream of GPAT, 1-acylglycerol-3-phosphate acyltransferase (AGPAT) catalyzes the addition of fatty acyl CoA to the *sn*-2 position of LPA, producing phosphatidic acid (PA). There are two major isoforms of AGPAT localized to the ER that have been well characterized, and as many as 10 have been reported in mammals (Takeuchi and Reue, 2009). CDP-diacylglycerol synthase (CDS) then

converts PA to CDP-DAG, the precursor to all membrane phospholipids (Horvath and Daum, 2013). A recent study revealed the first linkage between the synthesis of mitochondrial proteins and phospholipids. Knocking out PGC-1α and PGC-1β in mice resulted in a decrease in transcript levels of CDP-diacylglycerol synthase (CDS1) and levels of cardiolipin, phosphatidylcholine and phosphatidylethanolamine in hearts (Lai et al., 2014).

To shed light on how mitochondrial membrane synthesis is stimulated during cold-induced mitochondrial biogenesis in fish, we characterized the activity of GPAT and measured its activity in liver and oxidative muscle of threespine stickleback (*Gasterosteus aculeatus*) cold-acclimated to 8 °C and held at 20 °C. Previous studies in our lab have shown that mitochondrial density increases in oxidative pectoral adductor muscle but not liver in response to cold acclimation (Orczewska et al., 2010). We anticipated that if GPAT drives increases in mitochondrial membrane biosynthesis, GPAT activity would increase in oxidative muscle but not liver. Additionally, we measured transcript abundance of key genes of the phospholipid biosynthetic pathway, CDS1, CDS2, GPAT1 and AGPAT2, in liver and oxidative muscle of stickleback harvested during cold acclimation to 8 °C.

2.3 MATERIALS AND METHODS

2.3.1 *Animal care*

Threespine stickleback, *G. aculeatus*, were collected in August, 2012 and 2013 from Kashwitna Lake, AK (61°50' N, 150°00' W) using minnow traps. Fish were transported to UAF and maintained in 114 L aquaria filled with distilled water supplemented with 0.35% Instant Ocean, filtered and aerated.

Fish collected in 2012 were used to measure GPAT apparent Km, GPAT substrate preference, and maximum GPAT activity in frozen tissue. Fish for these studies were maintained on a 10 hr light, 14 hr dark cycle at 10°C and fed bloodworms daily for 9 months. Fish were euthanized with an overdose of tricaine methane sulfonate (MS-222) (250 mg L^{-}), pH 7 followed by cervical cord transection for measuring GPAT activity except for in frozen tissue. Fish for these assays were flash frozen in liquid nitrogen and stored at -80 °C for 35-45 days. All

protocols were approved by the University of Alaska Fairbanks (UAF) Institutional Animal Care and Use Committee (135490-2 and 135490-7).

Fish collected in 2013 were used for acclimation experiments. These animals were held at 20 °C for 20 weeks then cold-acclimated by decreasing the temperature in the environmental chamber where the fish were held to 15 °C on day 1, to 10 °C on day 2, and to 8 °C on day 3. Warm-acclimated fish were held at 20 °C in a separate room. Fish were held at 8 °C or 20 °C for an additional 11 wk for measuring GPAT activity. For measuring levels of mRNA, fish were harvested after 1 wk, 4 wk and 9 wk at 8 °C and 20 °C coinciding with the start of cold acclimation and after an additional 9 wk at 20 °C (at the end of cold acclimation). Animals were harvested each morning at the same time and prior to feeding. All were held on a 10 hr light, 14 hr dark cycle throughout acclimation. Fish were euthanized with an overdose of MS-222 (250 mg L^{-}), pH 7 followed by cervical cord transection for assaying GPAT activity and flash frozen in liquid nitrogen for measuring mRNA levels.

2.3.2 *Maximal GPAT activity of crude liver homogenate*

Liver and oxidative pectoral adductor muscle were excised from fish on ice and homogenized in 8 volumes of buffer (10 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, pH 7.4 at 4 °C) using a Tenboreck homogenizer. Tissues from two individuals were pooled to measure GPAT activity in warm- and cold- acclimated animals. Protein concentrations were determined using a Bradford assay with bovine serum albumin as a standard (Bradford, 1976). GPAT activity was carried out in a 500 µl reaction mixture containing 40 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 2 mg ml^{-} BSA (essentially fatty acid-free), 2 mM KCN, 100 µM of palmitoyl-CoA or palmitoleoyl-CoA, 0.1- 10 mM glycerol-3-phosphate and 0.1 µCi [^{4}C] glycerol-3-phosphate (G3P) (Perkin Elmer, Waltham MA, USA) (Vancura and Haldar, 1994). Reactions were initiated by the addition of 150-400 µg of protein. Maximal GPAT activity was linear for 20 min and proportional to the amount of homogenate added using palmitoyl-CoA as a fatty acyl donor (figures 2.A-1 and 2.A-2). The Km of GPAT was measured at 10 °C for 20 min using 400 µg of protein, 0.1-10 mM G3P and 100 µM of palmitoyl-CoA. The maximal activity of GPAT in fresh and frozen liver tissue was compared by measuring activity at 10 °C for 20

min using 400 μg of protein, 1 mM G3P and 100 μM of palmitoyl-CoA. Substrate preference was determined by measuring activity at 10 °C for 20 min using 400 μg of protein, 1 mM G3P, and 100 μM of palmitoyl-CoA or palmitoleoyl-CoA. GPAT activity in cold-acclimation experiments was assayed at 14 °C for 20 min in liver and pectoral adductor muscle from warm- and cold-acclimated animals in the presence of 5 mM of G3P, 100 μM of palmitoleoyl-CoA and 400 μg of protein. Reactions were stopped by the addition of 0.5 ml of H_2O-saturated 1-butanol followed by vortexing. Samples were centrifuged at 12,100X *g* for 5 min at room temperature. An aliquot of 200 μL of the butanol layer was added to 10 ml liquid scintillation fluid (Ecolite MP Biomedicals, LLC, Solon, OH, USA). ^4C was counted using an LS 6500 multi-purpose scintillation counter (Beckman Coulter, Fullerton CA, USA). Maximal activity of GPAT was measured in duplicate for measuring Km and substrate specificity, and in triplicate for measuring activity in frozen liver tissues and in warm- and cold-acclimated animals. Specific activity of GPAT was determined as nmol G3P acylated per min per mg protein (nmol min^- mg^-). Background activity was quantified in reaction mixtures lacking protein, and in acclimated animals, in reaction mixtures lacking palmitoleoyl-CoA.

2.3.3 *Gene expression*

RNA was isolated from 10-30 mg of oxidative pectoral adductor muscle and liver tissue from 6-8 individuals harvested at each time point during cold and warm acclimation using the RNeasy Fibrous Tissue Mini-kit (Qiagen) as described previously (Orczewska et al., 2010). RNA levels were quantified by measuring the absorbance at 260 nm with a Nano-Drop® (ND-1000) spectrophotometer. RNA integrity was verified by mixing 2 μl of RNA with loading buffer (5% glycerol, 0.04% bromophenol blue, 0.1 mmol l^{-1} EDTA, pH 8.0) and separated on a 2% agarose gel. Total RNA was reverse transcribed to complementary DNA (cDNA) using TaqMan® reverse transcription reagents (Applied Biosystems) as described previously (Orczewska et al., 2010). Briefly, cDNA was synthesized in a 10 μl reaction containing 5.5 mM $MgCl_2$, 2.5 μM random hexamers, 2 mM dNTPs, 4 U of RNase inhibitor, 37.5 U reverse transcriptase and 200 ng RNA. Synthesis reactions were performed using an iCycler (Bio-Rad Laboratories) programmed at 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min.

Transcript levels were measured in oxidative pectoral adductor muscle and liver tissue using quantitative real-time PCR (qRT-PCR) as described previously (Orczewska et al., 2010). Briefly, gene-specific primers were designed using sequence information obtained from Ensembl (www.ensembl.org) and the software Primer Express (Applied Biosystems) with at least one primer from each pair spanning a splice site to ensure that genomic DNA was not amplified (Table 2.1). Each primer was searched against the stickleback genome using the BLAST tool on Ensembl (www.ensembl.org) to ensure its specificity. Primers were synthesized commercially (Invitrogen). Transcript levels were measured using an ABI 7900HT sequence detection system (Applied Biosystems) in triplicate. 20 µl reaction mixtures contained 5 ng cDNA, 10 µl Power SYBR® Green Master Mix (Applied Biosystems) and 0.3 µM of each forward and reverse primer with the exception that 1 ng of cDNA was used in each reaction to quantify transcript levels of 18S. Optimal primer concentration was determined by measuring transcript levels using 0.2 µM, 0.3 µM and 0.4 µM of each forward and reverse primer. A dissociation curve was analyzed for each reaction to ensure specificity of each primer set. Two controls were used to identify and omit samples with contaminating genomic DNA. One control replaced the cDNA template with an equal volume of Milli Q H_2O. The second control was prepared for each sample during cDNA synthesis by omitting reverse transcriptase. Reaction efficiency and relative quantity of input RNA were determined using a standard curve for each gene prepared by pooling samples from warm- and cold- acclimated animals. The expression of each target gene was normalized to 18S in liver tissue and EF-1α in oxidative muscle; the expression of these genes was previously determined to remain constant in threespine stickleback during warm and cold acclimation (Orczewska et al., 2010).

2.3.4 *Statistical analyses*

One-way ANOVAs were used to determine differences in GPAT activity measured in the presence of differing concentrations of G3P, and in gene expression of GPAT1 and GPAT2. Differences in GPAT activity among cold- and warm- acclimated animals and tissue type were determined using a two-way ANOVA. ANOVAs were followed by a *post-hoc* Tukey's honestly significant difference (HSD). Data were normally distributed as determined by a Shapiro-Wilk test. Equal variance was confirmed with a Bartlett's test. Differences in GPAT activity between

measurements made with palmitoyl-CoA and palmitoleoyl-CoA and between fresh and frozen tissues were analyzed using a two-sample Student's *t* test. Data are presented as means ± s.e.m. Level of significance was set at $P < 0.05$. Statistical analyses were conducted in R 3.0.1 (R Core Development Team 2013,Vienna, Austria) with the additional package Sciplot version 1.1-0 (Morales, 2013) and with Sigma Plot v11.2 (Systat Software, San Jose, CA, USA) and JMP7 (SAS, Cary, NC, USA).

2.4 RESULTS

2.4.1 *Physical characteristics*

Fish used for measuring maximal GPAT activity showed no significant differences in length or body mass between fish held at 8 °C compared to those held at 20 °C (Table 2.2). Fish used for measuring mRNA levels also did not show a significant difference in length or body mass between fish held at 8 °C compared to those held at 20 °C (Table 2.3).

2.4.2 *Apparent Michaelis Menten constant*

Maximal GPAT activity measured in fresh liver tissue increased in the presence of G3P at concentrations above 0.1 mM but was not significantly different at concentrations between 1 mM and 10 mM G3P, indicating that GPAT is saturated at G3P concentrations equal to or greater than 1 mM ($P < 0.05$). Maximal activity of GPAT was 2.8-fold greater in the presence of 5 mM G-3P compared to 0.1 mM (0.5 nmol min^{-} mg^{-} protein ± 0.07 and 0.18 nmol min^{-} mg^{-} protein ± 0.03 respectively), and 2.4 greater in the presence of 10 mM compared to 0.1 mM G-3P (0.42 nmol min^{-} mg^{-} protein ± 0.04 and 0.18 nmol min^{-} mg^{-} protein ± 0.03 respectively). The apparent Km of G3P for GPAT was 0.21 mM, and maximum velocity (Vmax) was 0.33 nmol min^{-} mg^{-} protein (Fig. 2.1).

2.4.3 *Maximal GPAT activity in fresh and frozen tissue*

Maximal activity of GPAT was significantly lower in frozen liver tissue (0.32 nmol min^{-} mg^{-} protein ± 0.04) compared to fresh liver tissue (0.50 nmol min^{-} mg^{-} protein ± 0.07) when measured in the presence of 5 mM G3P and 100 μM palmitoyl-CoA ($P < 0.05$) (Fig. 2.2).

Results were based on the assumption that the Km for GPAT did not change in response to freezing the tissue.

2.4.4 *Substrate preference*

Maximal activity of GPAT measured in fresh liver tissue was not different in the presence of palmitoleoyl-CoA (0.76 nmol min^{-} mg^{-} protein ± 0.06) compared to palmitoyl-CoA (0.69 nmol min^{-} mg^{-} protein ± 0.09) when using 1 mM G3P ($P < 0.05$) (Fig. 2.3).

2.4.5 *GPAT activity in warm- and cold- acclimated stickleback*

Maximal activity of GPAT was 2.4 fold higher in in pectoral muscle of cold-acclimated animals compared to those held at 20 °C (0.29 nmol min^{-} mg^{-} protein ± 0.01 and 0.12 nmol min^{-} mg^{-} protein ± 0.03) (Fig. 2.4). There was no difference in GPAT activity in liver tissue between warm- and cold-acclimated individuals. Maximal GPAT activity in liver was 0.50 nmol min^{-} mg^{-} protein ± 0.03 in warm-acclimated fishes and 0.55 nmol min^{-} mg^{-} protein ± 0.04 in cold-acclimated fishes ($P > 0.05$). However, GPAT activity was higher in liver compared to pectoral muscle in both cold- and warm-acclimated animals ($P < 0.05$).

2.4.6 *Expression of genes involved in lipid biosynthesis*

Transcript levels of CDS 1 and CDS 2 did not change in response to cold acclimation in liver and CDS transcripts were undetectable in pectoral adductor muscle (Fig. 2.5A). GPAT1 transcripts increased in liver in animals held at 8 °C for 1 wk and then declined by wk 4, and was equivalent to animals held at 20 °C (Fig. 2.5B). AGPAT2 transcript levels did not change in liver during cold acclimation (Fig. 2.5B). In contrast, AGPAT2 mRNA levels increased in pectoral adductor in animals held at 8 °C for 1 wk and then declined and were equivalent to animals at 20 °C by wk 4 (Fig. 2.6). GPAT1 mRNA levels did not change in pectoral adductor muscle in response to cold acclimation (Fig. 2.6). GPAT2 mRNA was undetectable in liver and oxidative muscle.

2.5 DISCUSSION

Mitochondrial biogenesis during cold acclimation of fishes requires mitochondrial DNA replication, increased transcription and translation of mitochondrial proteins, and increased synthesis of phospholipids. Currently little is known about the molecular mechanisms governing phospholipid biosynthesis during cold acclimation but our studies suggest GPAT and/or AGPAT are upregulated in some tissues during cold acclimation and contribute to increased synthesis of phospholipids.

2.5.1 *Characterization of GPAT activity*

The apparent Km of G3P in GPAT measured at 10 °C in stickleback liver was 0.21 mM. Since we measured the maximal activity of GPAT in a crude homogenate, this reflects the average Km of all GPAT isoforms expressed in liver. In mammals, GPAT1 and GPAT4 are the primary isoforms expressed in liver but GPAT2 and GPAT3 are also expressed at low levels (Cao et al., 2006; Hammond et al., 2002; Wang et al., 2007). The Km of GPAT measured in microsomes of mice liver at room temperature is 0.14 mM, whereas the Km of mitochondrial isoforms was 0.4 mM (Lewin et al., 2004). The apparent Km for G3P in stickleback liver falls between the range of Kms in mice microsomal and mitochondrial isoforms. Although the Km values determined in mice were not measured at physiological temperature, but rather at room temperature, these Km values are similar to those we determined in stickleback and suggest affinity of the enzyme for G3P has been conserved.

Our results indicate that the pooled isoforms of GPAT in liver do not exhibit a preference for palmitoleoyl-CoA or palmitoyl-CoA. This conclusion is based on the assumption that Vmax is similar with each substrate and that each fatty acyl-CoA was in excess of its Km, which in mouse liver mitochondrial GPAT is between 3 and 15 μM (Vancura and Haldar, 1994). The Km for fatty acyl-CoAs of microsomal isoforms has not been described. We had predicted that GPAT might have a higher affinity for unsaturated fatty acyl CoAs than saturated ones because of the greater proportion of unsaturated fatty acids in membranes in cold-tolerant fishes, such as stickleback, compared to mammals (Logue et al., 2000). The four GPAT isoforms in mammals differ in their affinity for saturated and unsaturated fatty acyl chains. GPAT1 prefers saturated

fatty acyl CoAs, whereas GPAT2, 3 and 4 show no preference (Cao et al., 2006; Coleman and Lee, 2004; Lewin et al., 2004). The substrate preference of individual GPAT isoforms in fish remains unknown.

Increases in the maximal activity of GPAT in oxidative muscle of stickleback in response to cold acclimation is correlated with increases in mitochondrial density. The maximal activity of GPAT increased 2.4-fold in response to cold acclimation in the pectoral muscle of *G. aculeatus*, coinciding with a 1.9-fold increase in mitochondrial density (Orczewska et al., 2010). This correlation suggests that GPAT activity is involved in mitochondrial membrane biogenesis and may contribute to phospholipid synthesis during cold acclimation in stickleback.

The higher maximal activity of GPAT in liver tissue compared to oxidative skeletal muscle likely reflects the dual role of GPAT in synthesizing both phospholipids and TAGs. TAGs occupy 57 – 59% of hepatocyte volume compared to only 0.4 - 0.5% in oxidative pectoral adductor muscle (Orczewska et al., 2010). The density of lipid droplets does not change in either pectoral adductor muscle or liver in response to cold acclimation, suggesting that the increase in GPAT activity in pectoral adductor likely contributes to an increase in mitochondrial density in oxidative skeletal muscle, rather than increased synthesis of TAGs (Orczewska et al., 2010).

2.5.2 *Molecular drivers of membrane biosynthesis*

Little is known about what regulates mitochondrial membrane phospholipid biosynthesis during mitochondrial biogenesis. A recent study in mammals showed that mRNA levels of CDP-diacylglycerol synthase (CDS1), which catalyzes the synthesis of CDP- diacylglycerol (CDP-DAG from PA and CTP, is regulated by PGC-1α, providing the first linkage between the regulation of the synthesis of mitochondrial proteins and lipids (Lai et al., 2014). However, in our study, neither CDS1 nor CDS2 increased in response to cold acclimation in either liver or pectoral adductor muscle, and transcripts were undetectable in oxidative pectoral adductor muscle. In mice, knocking out PGC-1α and PGC-1β led to downregulation of CDS1, and a decrease in cardiolipin in cardiac myocytes, suggesting CDS1 is critical for cardiolipin biosynthesis (Lai et al., 2014). The lack of detectable levels of CDS1 and 2 in oxidative pectoral adductor muscle is surprising. The synthesis of CDP-DAG is essential not only for the

production of cardiolipin, but also phosphatidylglycerol, and phosphatidylinositol (Horvath and Daum, 2013). How the synthesis of these phospholipids is regulated in fish muscle warrants further study.

The lack of change in CDS in stickleback, particularly in muscle where mitochondrial biogenesis occurs, may be due to differences in the pathway mediating mitochondrial biogenesis between fish and mammals. In fish, and in contrast to mammals, PGC-1α is not the master regulator of mitochondrial biogenesis. PGC-1α levels do not change in response to cold acclimation in muscle of goldfish (Bremer et al., 2012; LeMoine et al., 2008), zebrafish (McClelland et al., 2006) or stickleback (Orczewska et al., 2010) or in response to exercise in zebrafish (McClelland et al., 2006). Comparison of PGC-1α sequences among vertebrates revealed a polyserine insertion within the NRF-1 binding domain of PGC-1α in teleosts, which may eliminate its capacity to mediate mitochondrial biogenesis (LeMoine et al., 2008). Indeed, NRF-1 appears to play a more prominent role in inducing mitochondrial biogenesis in fish, as NRF-1 mRNA levels increase in response to both exercise and cold temperature in several species (Bremer et al., 2012; LeMoine et al., 2008; McClelland et al., 2006; Orczewska et al., 2010). AGPAT mRNA levels increase after 1 week of cold acclimation in stickleback, concordantly with increases in NRF-1 mRNA levels, suggesting NRF-1 may induce AGPAT expression, contributing to an increase in mitochondrial membrane biosynthesis.

In conclusion, results from this study suggest that cold-acclimated threespine sticklebacks (*G. aculeatus*) increase activity and transcript levels of enzymes glycerol-3-phosphate acyltransferase (GPAT) and/or 1-acylglycerol-3-phosphate transferase (AGPAT) of the lipid synthesis pathway in oxidative muscle, which may contribute to mitochondrial phospholipid biosynthesis during mitochondrial biogenesis. Overall, GPAT activity was higher in liver of stickleback but increased in response to cold acclimation only in pectoral adductor muscle, where mitochondrial biogenesis occurs (Orczewska et al., 2010). In contrast, transcript levels of GPAT1 increased in liver in response to cold acclimation, and not in pectoral adductor, and transcript levels of AGPAT2 increased in pectoral adductor but not liver. These data suggest two possibilities. First, protein levels may not change concordantly with mRNA levels. Alternatively, the activity we measured as GPAT may include both GPAT and AGPAT activity. AGPAT

catalyzes the addition of fatty acyl CoA to the *sn*-2 position of glycerol-3 phosphate, producing phosphatidic acid (PA). Consistent with this, previous studies have reported a mixture of LPA and PA as products of the assay we used to measure the activity of GPAT (Vancura and Haldar, 1992).

2.6 ACKNOWLEDGEMENTS

Funding for this research was provided by a grant from the *National Science Foundation* to KOB (IOS-0643857).

2.7 FIGURES

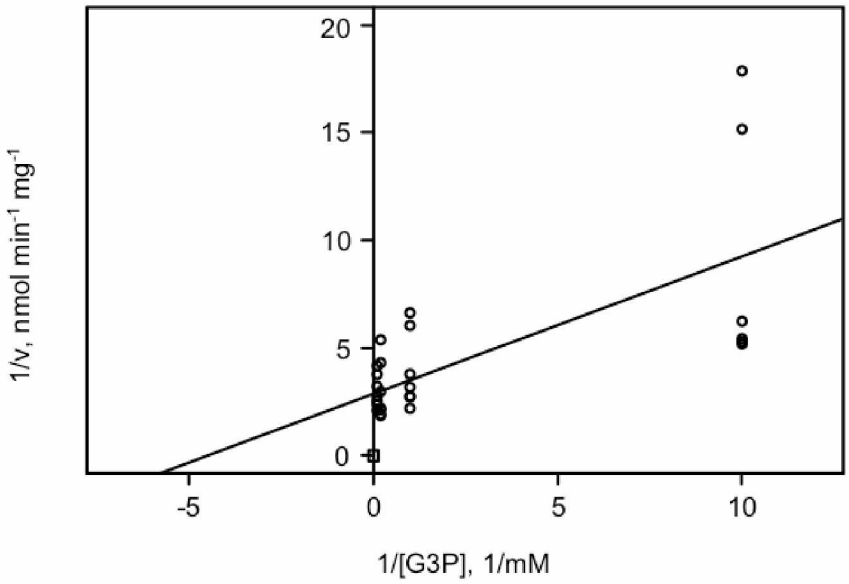

Fig. 2.1. Lineweaver-Burk plot of GPAT activity in fresh liver tissue. Activity was measured with 0.1-10 mM G3P and 100 μM palmitoyl- CoA. Circles represent reciprocal velocities at each reciprocal substrate concentration of G3P (10,1,0.2, and 0.1 mM), the square marks the origin and v on y-axis indicates velocity. The Km is calculated from the x-intercept (-1/Km) and Vmax from the y-intercept (1/Vmax).

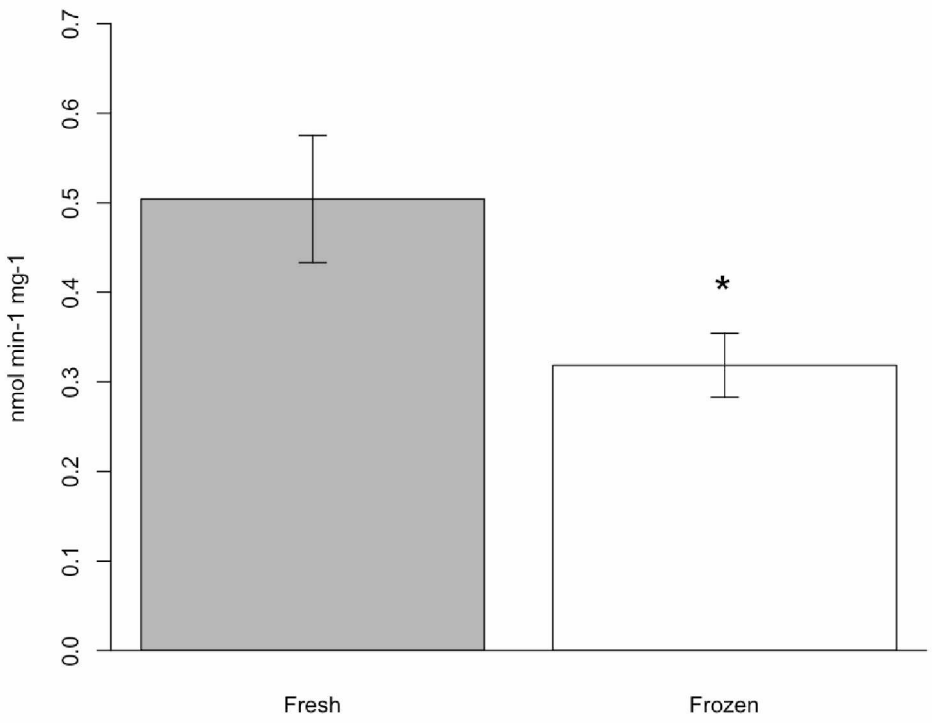

Fig. 2.2. Maximal GPAT activity in *G. aculeatus* fresh and frozen liver. Activity was measured fresh (N=9) and frozen (N=7) liver tissue in the presence of 5 mM G-3P and 100 μM palmitoyl-CoA. Asterisk indicates a significant difference ($P < 0.05$). Values represent the means ± s.e.m.

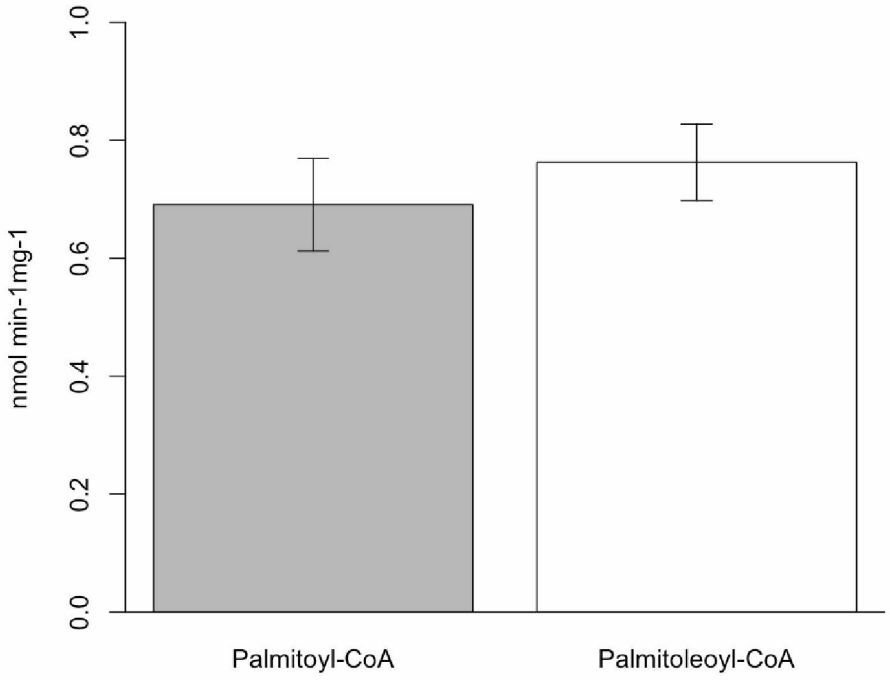

Fig. 2.3. Fatty acyl substrate preference of GPAT in *G. aculeatus*. Maximal total GPAT activity in *G. aculeatus* fresh liver tissue in the presence of 1 mM G3P and 100 μM fatty acyl donors palmitoyl-CoA, (N=5) and palmitoleoyl-CoA (N=7). Values represent the means ± s.e.m.

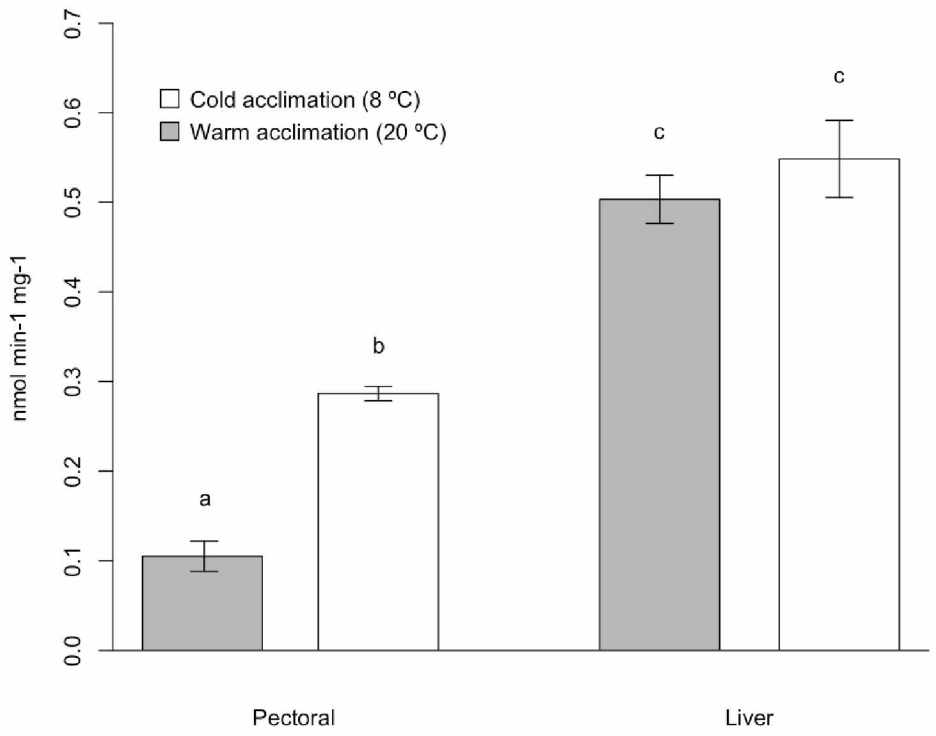

Fig. 2.4. GPAT activity in pectoral adductor and liver tissue of cold-acclimated *G. aculeatus*. Maximal GPAT activity was measured in liver tissue and pectoral muscle of warm (20 °C) and cold-acclimated (8 °C) *G. aculeatus* (N=6) in the presence of 5 mM G-3P and 100 µM palmitoleoyl-CoA. Significant differences are indicated by different letters. Values represent the means ± s.e.m ($P<0.05$).

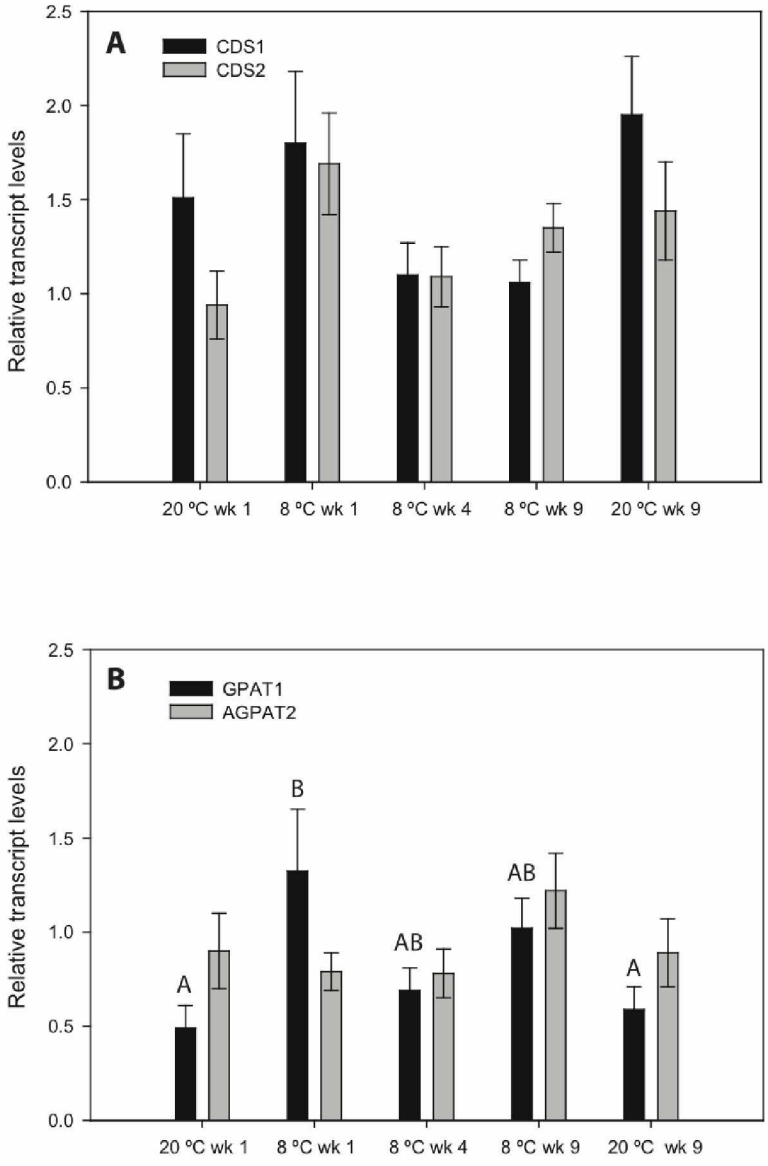

Fig 2.5. Transcript levels of genes involved in membrane biosynthesis in liver. Transcripts of **A** CDS1 and CDS2 and in **B** GPAT1 and AGAT2 measured in stickleback during cold acclimation. Different letters indicate significant differences among fish harvested at different times and temperature during acclimation ($P < 0.05$).

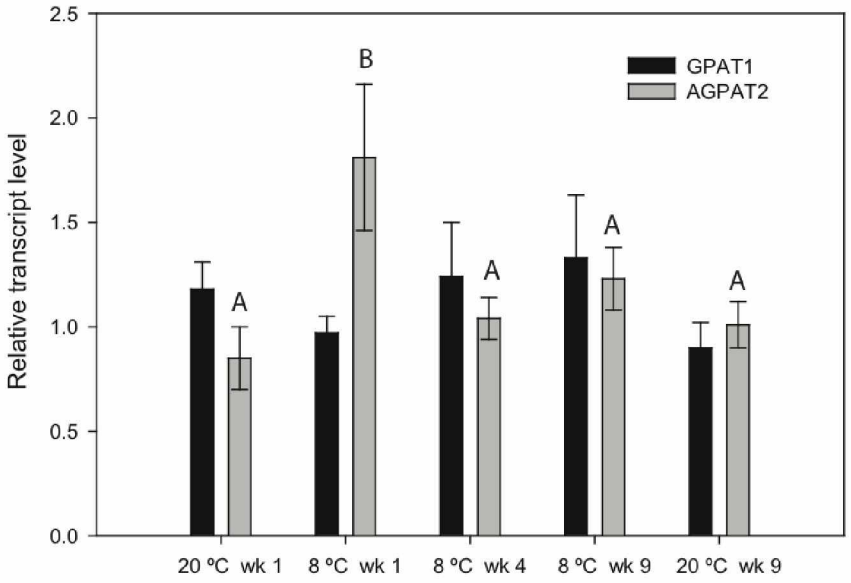

Fig. 2.6. Transcript levels of genes involved in membrane biosynthesis in oxidative muscle. Transcripts measured in pectoral adductor muscle of stickleback during cold acclimation. Different letters indicate significant differences among fish harvested at different times and temperature during acclimation ($P < 0.05$).

2.8 TABLES

Table 2.1.
Sequences of primers used for qRT-PCR

Gene	Sequence (5' to 3')	Amplicon size (bp)
AGPAT2	F ATCATCTCCAACCACCAGAGCT	51
	R GATCTCCATCAGGCCCAACA	
CDS1	F CAGTTCCTGGCGCGCTAT	51
	R TGCGAGGTACAAGGCGAAG	
CDS2	F CTCTTCGCATCCTCAGCAAATAC	51
	R GGTAGAGGGCAAAGGAGATGAAT	
GPAT1	F ACAGCAATGGCCTTTTCCAC	51
	R TGCAAGCAATGATTGCATCAG	
GPAT2	F CCCGGATAGAAGCGTGAGG	51
	R TCCTTGAGAGAAAAGGGCTGAG	

F = forward primer, R= reverse primer

Table 2.2. Effects of cold temperature on physical characteristics (GPAT assays). *G. aculeatus* was used for acclimation GPAT assays.

Temperature	*N*	Length, cm	Body mass, g
8° C	12	5.59 ± 0.14	1.53 ± 0.08
20° C	12	5.77 ± 0.12	1.65 ± 0.08

Values are means ± s.e.m. *N*= number. of fishes

Table 2.3. Effects of cold temperature on physical characteristics (mRNA levels). *G. aculeatus* was used for measuring mRNA levels of genes involved in phospholipid biosynthesis.

Temperature and Time Point of Harvest	***N***	**Length, cm**	**Body mass,g**
8°C *week 1*	16	4.94 ± 0.06	1.24 ± 0.06
8°C *week 4*	11	5.14 ± 0.13	1.44 ± 0.11
8°C *week 9*	9	5.41 ± 0.07	1.60 ± 0.08
20°C *week 1*	10	5.18 ± 0.13	1.41 ± 0.10
20°C *week 9*	10	5.45 ± 0.10	1.59 ± 0.10

Values are means ± s.e.m. *N*= number of fishes.

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response to cold acclimation. Am. J. Physiol. Regul. Integr. Comp. Physiol. 299, R352-364.
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APPENDIX 2.A

SUPPLEMENTAL FIGURES

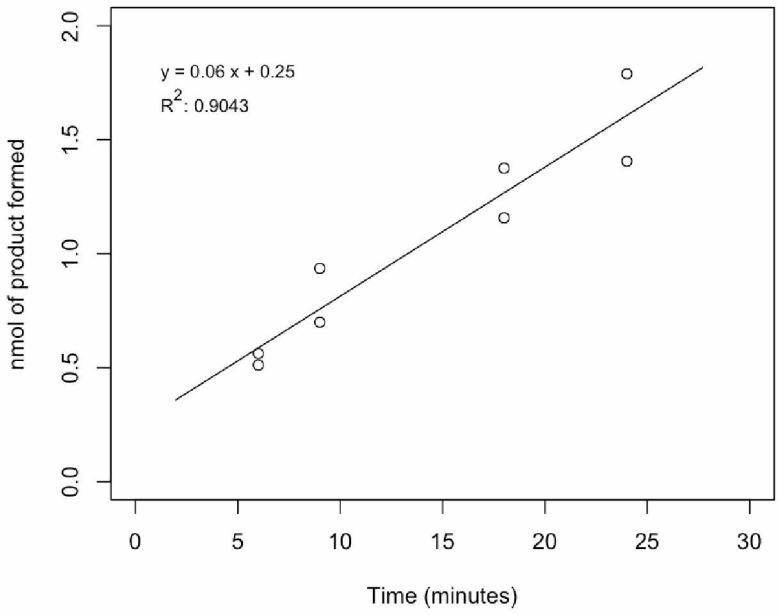

Fig. 2.A-1. GPAT activity is linear over time. GPAT activity measured in *G. aculeatus* fresh liver with 400 µg of protein at 6, 9, 18 and 24 min N=2.

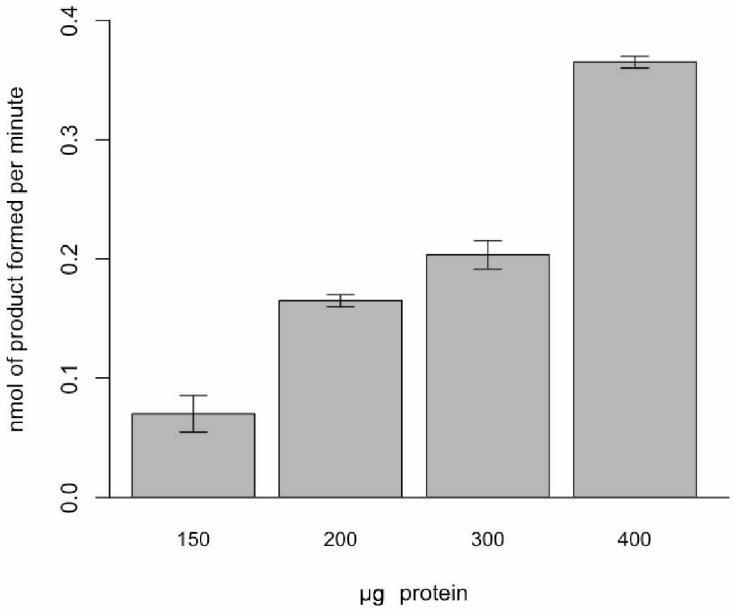

Fig. 2.A-2. GPAT activity is proportional to the amount of protein. GPAT activity was measured with 150, 200, 300 and 400 µg of liver protein with 100 µM palmitoyl-CoA and 1 mM G3P (N=5).

APPENDIX 2.B

PERMISSION OF COAUTHORS FOR PUBLICATION

Permission granted from co-author Megan Hoffman:

To Whom It May Concern,
I, Megan Hoffman, assisted Kelly Keenan with a portion of the work included in her thesis and hereby give her permission to include any or all of my contribution.
Sincerely,
Megan Hoffman

Permission granted from co-author Kristin Dullen:

I, Kristen Dullen, give Kelly Keenan permission to include my research data in her thesis.

GENERAL CONCLUSION

My work characterized the role of the enzyme glycerol-3-phosphate acyltransferase (GPAT) in regulating the synthesis of mitochondrial membranes during cold-induced mitochondrial biogenesis in oxidative muscle of the temperate fish species, *Gasterosteus aculeatus*, and the role of GPAT in maintaining high mitochondrial lipid densities in hearts of Antarctic fishes.

The maximal activity of GPAT increased 2.4-fold in response to cold acclimation in the pectoral muscle of *G. aculeatus*, coinciding with a 1.9-fold increase in mitochondrial density in oxidative muscle, suggesting that GPAT activity is involved in mitochondrial membrane biogenesis (Orczewska et al., 2010). However, the activity measured with our assay may include both GPAT and 1-acylglycerol-3-phosphate acyltransferase (AGPAT) activity. AGPAT operates downstream of GPAT, converting LPA (the product of GPAT) to phosphatidic acid (PA), the precursor to all phospholipids and triacylglycerides. Nevertheless, lipid synthesis is increased during cold acclimation. Without fractionating the lipid products of the assay, however, it can not be stated with certainty whether GPAT and/or AGPAT drives increases in mitochondrial membrane synthesis in response to cold acclimation.

Changes in the maximal activity of GPAT activity and transcript levels of GPAT1 and GPAT2 did not correspond in stickleback in response to cold acclimation. Similarly, GPAT1 and GPAT2 mRNA levels were not correlated with mitochondrial density and higher in hearts of icefishes compared to red-blooded fishes. These data suggest several possibilities. First, mRNA levels may not reflect protein levels. Alternatively, post-translational modifications of GPAT may regulate its activity. Another possibility is that AGPAT, the enzyme subsequent to GPAT, may contribute to increases in lipid synthesis during mitochondrial biogenesis. AGPAT2 transcript levels increased in pectoral adductor muscle in stickleback in response to cold acclimation and, GPAT activity measured in stickleback may represent the sum of activities of both GPAT and AGPAT. Lastly, GPAT3, which was not measured in this study, may regulate mitochondrial phospholipid synthesis.

Future studies ought to measure maximal activity of individual isoforms of GPAT and AGPAT in cellular fractions (mitochondrial and microsomal) followed by thin-layer chromatography to identify lipid products so to determine if GPAT and/or AGPAT and which isoforms of these enzymes increase in response to cold acclimation and if the activity of these enzymes differs between red- and white-blooded notothenioids. AGPAT2 transcript levels could also be measured in hearts of red- and white-blooded notothenioids. These data would clarify how high densities of mitochondrial phospholipids are maintained in oxidative muscles of icefishes and which enzymes(s) are upregulated during cold-induced mitochondrial biogenesis in fishes.

Finally, mine is the first study to sequence full-length cDNA of a GPAT isoform in a cold-adapted species and provide insight to the evolution of a mitochondrial membrane-bound enzyme in Antarctic and sub-Antarctic notothenioids. Antarctic notothenioids inhabit the Southern Ocean where temperatures range between -1.8 and 2 °C; the sub-Antarctic species *E. maclovinus* inhabits much warmer waters along the Argentinian Patagonian shelf and the Pacific Coast (4-10 °C) (Andriashev, 1965; Eastman, 1993; Logue et al., 2000; Norman et al., 1937; Regan, 1914). My study revealed that the cDNA sequence of GPAT1 is highly conserved between fishes from these two thermally different habitats. GPAT1 cDNA sequences show 98.4% identity among the Antarctic notothenioids *C. aceratus,* and *N. coriiceps*, and 96-96.1% identity between the two Antarctic species and the sub-Antarctic notothenioid, *E. maclovinus*. There were three differences in the amino acid sequence of GPAT1 between *E. maclovinus* and the two Antarctic species: Ser415Ala, Asp603Glu and Thr648Ala. I predicted greater differences in GPAT1 between Antarctic and sub-Antarctic notothenioids considering that molecular phylogenies indicate that *E.maclovinus* separated from other notothenioids between 22 and 23 million years ago and has since remained in warmer waters (Bargelloni and Lecointre, 1998). The high similarity of GPAT1 between the two Antarctic species and the sub-Antarctic notothenioid *E. maclovinus* indicates that the gene is under negative, or purifying, selection resulting in low variation in this gene among these notothenioids. GPAT is considered the rate-limiting enzyme in glyerolipid synthesis, therefore it is conceivable that it would demonstrate low variation, supporting its highly conserved function in metabolism and membrane synthesis. All three notothenioid species show 100% identity in active site motifs I-IV. This was expected

because active sites are generally highly conserved among warm- and cold-adapted homologues because enzymes must maintain a specific geometry for catalysis and substrate binding to conserve protein function (Crawford et al., 1989; Fields and Houseman, 2004; Fields and Somero, 1998; Jaenicke, 1991).

Comparison of GPAT1 between the two Antarctic species and the sub-Antarctic species revealed 100% conservation in transmembrane domain I and II. The high percentage of identity of transmembrane domains was surprising because membranes of the Antarctic species are expected to be more highly unsaturated than *E. maclovinus* due to the inverse relationship between habitat temperature and percentage of unsaturation in fatty acids in membrane phospholipids of fishes (Logue et al., 2000). Other studies of membrane-bound proteins in Antarctic fishes demonstrate how amino acid sequences can differ in transmembrane domains and likely facilitate protein insertion into highly unsaturated membranes. For example, Cys67 and Cys83 in Antarctic fishes are thought to form a disulfide bond within the transmembrane domain of Δ^9 desaturase. *E. maclovinus*, having a less unsaturated membrane, replaces Cys with Leu67 (Porta et al., 2013).

Functional studies are necessary to determine how amino acid substitutions in notothenioids may affect function of GPAT1. Future studies could include conducting mutagenesis experiments to determine whether substitutions in GPAT1 of the Antarctic notothenioids enhance function at chronically cold temperatures, rendering the enzyme cold-adapted. It would also be interesting to determine which amino acids within the C-terminal region of GPAT1 in fishes are critical for enzyme activity, for fishes have an additional five amino acid sequence in this domain not found in birds or mammals. In mammals, studies have shown that the entire C-terminal domain (amino acids 594-828) of GPAT1 is necessary for activity and interacts with the N-terminal domain in the cytosol (Pellon-Maison et al., 2006).

GENERAL CONCLUSION REFERENCES

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